EFFECTS OF HIGH-DENSITY LIPOPROTEIN FROM PATIENTS WITH CHRONIC KIDNEY DISEASE ON ENDOTHELIAL FUNCTION

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Thimoteus Speer

aus Deutschland

Promotionskomitee

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Zürich, 2013

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To my parents

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1 Zusammenfassung

Die chronische Niereninsuffizienz (CKD) stellt einen bedeutenden Risikofaktor für die Entstehung kardiovaskulärer Ereignisse dar. Bereits eine nur leichtgradig eingeschränkte Nierenfunktion ist bereits mit einem erhöhten kardiovaskulären Risiko assoziiert. High-density Lipoprotein (HDL) von Gesunden weist neben seiner Rolle im reversen Cholesterintransport bedeutende antiatherogene Eigenschaften auf. Allerdings zeigen aktuelle Studien, dass die vaskulären Effekte von HDL sehr heterogen sein können. Ziel des Projektes war es, die vasoprotektiven Eigenschaften von HDL von Patienten mit einer chronischen Niereninsuffizienz zu untersuchen.

Im Gegensatz zu HDL von Gesunden ($HDL^{Healthy}$) führte die Inkubation von humanen aortalen Endothelzellen (HAEC) mit HDL von Nierenkranken (HDL^{CKD}) zu einer dramatischen Hemmung der endothelialen Stickstoffmonoxid (NO) Produktion. Dieser Effekt war bereits mit HDL von Patienten mit nur leichtgradig eingeschränkter Nierenfunktion zu beobachten. Im Mausmodell führte eine Injektion von HDL^{CKD} zu einem Blutdruckanstieg, während $HDL^{Healthy}$ den Blutdruck reduzierte. Ferner konnten wir zeigen, dass HDL^{CKD} in HAEC die Produktion von Superoxid-Radikalen stimuliert. Mittels Massenspektromie konnten wir die Akkumulation von symmetrischem Dimethylarginin (SDMA) in HDL^{CKD} aber nicht in $HDL^{Healthy}$ nachweisen. Außerdem konnten wir zeigen, dass die Inkorporation von SDMA in den HDL (HDL^{SDMA}) Partikel zur Veränderung der endothelialen Eigenschaften von HDL führt. HDL^{SDMA} hemmte die endotheliale NO-Produktion und förderte die Produktion von Superoxid-Radikalen. Weiter konnten wir nachweisen, dass die Effekte von HDL^{CKD} als auch HDL^{SDMA} durch eine Aktivierung des Toll-like Rezeptors-2 (TLR-2) auf der Oberfläche von Endothelzellen vermittelt werden. Im Gegensatz zu klassischen TLR-2 Liganden, die via TLR-2 zusammen mit den Co-Rezeptoren TLR-1 und TLR-6 die NF-κB abhängige Cytokinproduktion stimulieren, konnten wir erstmalig aufzeigen, dass für die TLR-2-abhängige Aktivierung von Endothelzellen durch HDL^{CKD} und HDL^{SDMA} diese Co-Rezeptoren nicht notwendig sind. HDL^{CKD} und HDL^{SDMA} führen beiden über eine Aktivierung des endothelialen TLR-2 zu einer direkten Hemmung der stimulatorischen Akt-abhängigen Phosphorylierung der endothelialen NO Synthase (eNOS) sowie via Phosphorylierung von SAPK/JNK zu einer NADPH-Oxidase abhängigen Produktion von Superoxid-Radikalen. Schließlich konnten wir nachweisen, dass die Reduktion der endothelialen NO-Bioverfügbarkeit durch HDL^{CKD} und HDL^{SDMA} auch zu einer Aufhebung der anti-inflammatorischen Eigenschaften von HDL und der positiven Eigenschaften des HDL auf die Reparatur von Endothelläsionen führt.

Zusammenfassend konnten wir erstmals zeigen, dass HDL von Nierenkranken endothelialer Dysfunktion sowie einen Anstieg des Blutdrucks induziert. Wir konnten ferner nachweisen,

dass die Akkumulation von SDMA im HDL von Nierenkranken die Umkehr der physiologischen Eigenschaften des HDLs hin zu einem gefäßschädigenden Partikel induziert. Insgesamt zeigen diese Ergebnisse einen neuen Zusammenhang zwischen endothelialer Dysfunktion, arterieller Hypertonie sowie dem angeborenen Immunsystem auf.

2 Summary

Chronic kidney disease (CKD) represents a potent cardiovascular risk factor. Thereby, a slightly reduced renal function is already associated with an elevated risk for cardiovascular events. Besides its role in the reverse cholesterol transport, high-density lipoprotein (HDL) from healthy subjects exerts several important vascular effects. However, recent evidence suggests that the vascular properties of HDL can be highly heterogeneous. The aim of the present project was to examine the vasoprotective effects of HDL from patients with CKD.

In contrast to HDL from healthy subjects ($HDL^{Healthy}$), incubation of human aortic endothelial cells (HAEC) with HDL from patients with CKD (HDL^{CKD}) markedly inhibited endothelial nitric oxide (NO) production. Interestingly, this effect was already present with HDL from patients with incipient CKD. In a mouse model, HDL^{CKD} increased the arterial blood pressure, whereas $HDL^{Healthy}$ reduced the arterial blood pressure. Moreover, we could show that HDL^{CKD} induced the production of superoxide radicals in HAEC. Using a mass-spectrometry approach, we identified the accumulation of symmetric dimethylarginine (SDMA) in HDL^{CKD}, but not in $HDL^{Healthy}$. Moreover, we could demonstrate that incorporation of SDMA into HDL (HDL^{SDMA}) inverts the vasoprotective properties of HDL. HDL^{SDMA} inhibited endothelial NO production and stimulated superoxide production in HAEC. Subsequently, we could reveal that the adverse vascular effects of $HDL^{Healthy}$ as well as HDL^{SDMA} are mediated by Toll-like receptor-2 (TLR-2) on the surface of endothelial cells. In contrast to classical TLR-2 ligand, in which hetero-dimers of TLR-1/2 or TLR2/6 mediate NF-κB-dependent cytokine secretion, we could show for the first time that TLR-2 dependent endothelial activation by HDL^{CKD} or HDL^{SDMA} is co-receptor independent. Moreover, we found that activation of endothelial TLR-2 by HDL^{CKD} or HDL^{SDMA} directly reduces the Akt-dependent stimulatory phosphorylation of endothelial NO synthase (eNOS) and stimulates the NADPH-oxidase dependent endothelial superoxide production via phosphorylation of SAPK/JNK. Moreover, our results indicate that the reduced endothelial NO bioavailability in response to HDL^{CKD} or HDL^{SDMA} suppresses the anti-inflammatory properties of HDL as well as the effects of HDL on endothelial repair.

In summary, we could show for the first time that HDL^{CKD} induces endothelial dysfunction and increases arterial blood pressure. Moreover, we identified SDMA in the HDL fraction as the culprit transforming HDL^{CKD} from a vasoprotective into a noxious particle. Of note, these findings reveal a novel link between endothelial dysfunction, hypertension and innate immunity.

3 Introduction

3.1 Atherosclerotic diseases as main cause of death in western populations

Atherosclerotic disease such as myocardial infarction, stroke and peripheral artery disease represent the main cause of death in western populations. In 2008, the prevalence of cardiovascular disease in the United States was 36.2 % affecting approximately 80.000.000 US citizens. Moreover, cardiovascular disease was responsible for almost 6.000.000 hospital admissions leading to healthcare costs of $ 286.6 Billion. Although the mortality declined during the last years, the prevalence of cardiovascular disease remains high (Roger et al. 2011), presumably because of an increasing prevalence of classical cardiovascular risk factors such as age, male sex, diabetes mellitus, hypertension, smoking and hyperlipidemia as identified by the Framingham Heart Study (Kannel et al. 1961).

3.1.1 Pathogenesis of atherosclerosis

Atherosclerosis is characterized by atheromatous plaques developing in the inner surface of arteries, which can disrupt and then promote the formation of thrombi. Subsequently, these thrombi may lead to flow-limiting stenoses of the blood vessels causing tissue ischemia, which clinically manifests as myocardial infarction or stroke. The process of atherogenesis is depicted below (Figure 1).

The normal endothelium represents a barrier preventing activation and adhesion of circulating vascular cells such as mononuclear cells (MNC), lymphocytes or platelets. Once activated by a variety of stimuli (e.g. tumor necrosis factor α [TNF-α], interleukin 1β [IL-1β], angiotensin-2, modified low-density lipoprotein [LDL]), endothelial cells express cell adhesion molecules (CAM; e.g. intercellular adhesion molecule [ICAM], vascular cell adhesion molecule 1 [VCAM-1] or selectins) and secrete proinflammatory cytokines, which promote chemotaxis and adhesion of circulating vascular cells to the endothelial monolayer (Weber et al. 2008; Hansson et al. 2011). Besides, changes of the endothelial permeability promote the accumulation of lipids and lipoproteins in the subendothelial layer (Tabas et al. 2007). Subsequently, MNC can transmigrate into the subendothelial layer, where they are activated to differentiate into macrophages. There, macrophages engulf lipids and lipoproteins and become foam cells. Moreover, vascular smooth muscle cells (VSMC) migrate from the media into intima and start to proliferate. They release extracellular matrix constituents such as collagen and elastin, which form a fibrous cap covering the plaque. Thereby, the plaque consists of macrophage-derived foam cells, extracellular lipid deposits, cholesterol crystals and apoptotic cell debris, which can not be efficiently removed leading to the formation of a necrotic core of the plaque (Tabas 2010). After physical disruption of the fibrouse cap,

extracellular matrix constituents are exposed to blood, which induces the activation of the plasmatic coagulation cascade and platelets leading to the formation of thrombi.

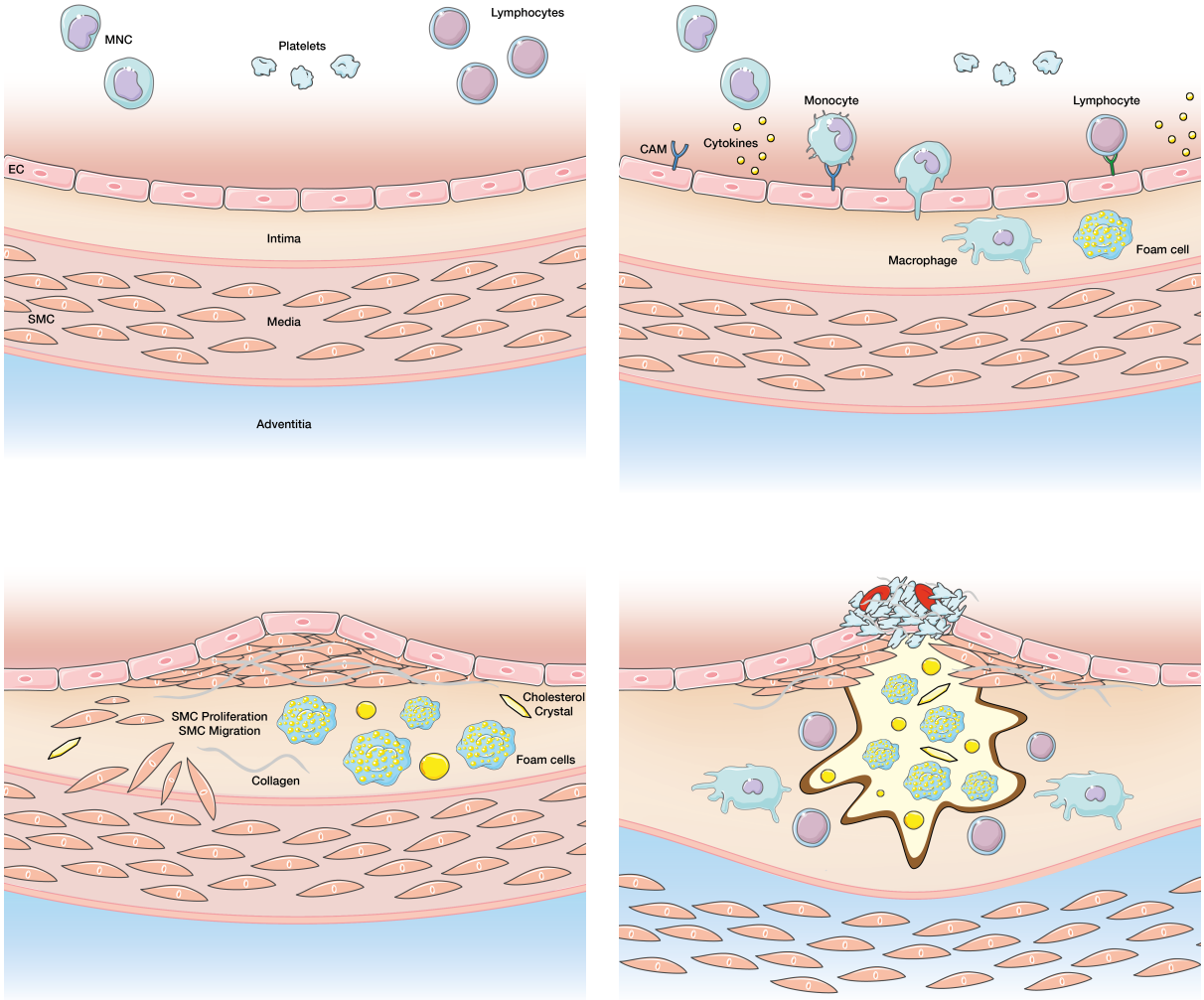

Figure 1 – Process of atherogenesis

3.1.2 Endothelial dysfunction – the promoter of atherogenesis in CKD

The endothelium is the largest organ of the human body consisting of approximately 10^{13} cells with a total surface of roughly 1.000 m^2. Endothelial cells cover the vasculature as a barrier between the blood stream and the vessel wall. However, the endothelium is rather a complex control center than a simple physical barrier. Endothelial cells are key regulators of the vascular homeostasis: they control vascular tone, modulate inflammation of the vessel wall, equilibrate platelet activation and hemostasis, thereby interacting with other cell types of the vessel wall (e.g. vascular smooth muscle cells, macrophages) and circulating blood cells (e.g. mononuclear cells, platelets) (Fleming et al. 2003; Rao et al. 2007; Munzel et al. 2008). To regulate vascular function and structure, endothelial cells produce a broad variety of anti-

atherosclerotic substances, of which nitric oxide (NO) is the best characterized and most studied. Generated by endothelial NO synthase (eNOS), NO induces relaxation of vascular smooth muscle cells, prevents expression of vascular cell adhesion molecules and adhesion of leukocytes, and inhibits platelet activation (Feletou et al. 2011).

Function	**Factor / Mediator**
Regulation of vascular tone	● Nitric oxide ● Cyclooxygenase derived products (e.g. prostacyclin) ● Endothelial-derived hyperpolarizing factor (EDHF) ● Endothelial-derived contracting factor (EDCF) ● Endothelin-1 ● Angiotensin II ● C-type natriuretic peptide ● Bradykinin
Regulation of thrombosis and hemostasis	● Nitric oxide ● Tissue plasmin activator (tPA) ● Thrombomodulin ● Prostaglandin ● Plasminogen activator inhibitor-1 (PAI-1) ● Tissue factor (TF) ● Tissue factor pathway inhibitor (TFPI) ● Platelet activating factor (PAF) ● Von Willebrand's factor (vWF)
Regulation of vascular inflammation	● Monocyte chemotactic factor-1 (MCP-1) ● Cell adhesion molecules (VCAM-1, ICAM-1, E-selectin) ● Interleukin 1, 6, 18 ● Tumor necrosis factor α
Regulation of cell proliferation	● Nitric oxide ● Transforming growth factor-β ● Endothelin-1 ● Angiotensin II ● Platelet derived growth factor (PDGF) ● Basic fibroblast growth factor ● Insulin like growth factor

Table 1 – Vascular function affected by endothelial cells and selected mediators

Deterioration of these beneficial endothelial effects (endothelial dysfunction) is thought to be the first critical step in the pathogenesis of atherosclerosis (Ross 1999).

A widely used definition of endothelial dysfunction refers to a dysbalance between the production of vasodilatory and vasoconstrictive substances by the endothelium. However, endothelial dysfunction may include deterioration of more endothelial properties than dysregulation of the vascular tone. Accordingly, endothelial dysfunction can generally be defined as an impairment of endothelial functional properties leading to abnormal endothelial activation.

Abnormal endothelial vasomotor function

The regulation of the vascular tone in cross-talk with vascular smooth muscle cells (VSMC) is a key feature of the endothelium. This is achieved by secretion of vasodilatory substances such as NO, prostacyclin and endothelial-derived hyperpolarization factor (EDHF), which induce relaxation of VSMC. However, in chronic kidney disease (CKD) several substances like homocysteine, endogenous methylarginines (e.g. asymmetric and symmetric dimethylarginine), or modified lipoproteins inhibit eNOS resulting in a reduced NO bioavailability. Moreover, some of them activate endothelial oxidases (e.g. NADPH oxidase, xanthine oxidase) to produce reactive oxygen species (ROS), which can directly act on vascular smooth muscle cells, react with NO to form the peroxynitrite radical or induce uncoupling of eNOS to produce ROS. In addition, cyclooxygenase-derived factors and endothelin-1 are released, causing a contraction of the smooth muscle cells together with ROS (Forstermann 2008; Shi et al. 2009; Feletou, Kohler et al. 2011; Vanhoutte 2011).

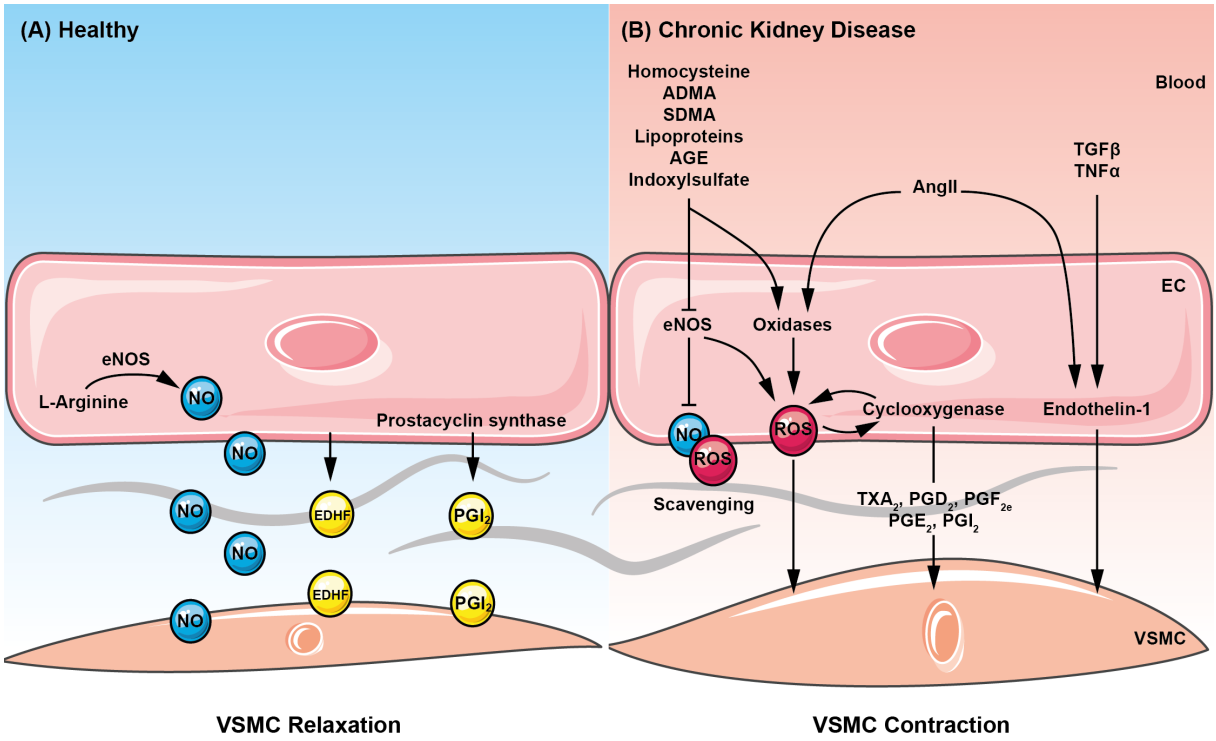

Figure 2 - Crosstalk between endothelial cells (EC) and vascular smooth muscle cells (VSCM) in health and chronic kidney disease. (A) In healthy subjects, endothelial-derived factors like nitric oxide (NO), endothelial-derived hyperpolarizing factor (EDHF) and prostacyclin (PGI_2) induce relaxation of vascular smooth muscle cells. (B) In chronic kidney disease, several substances inhibit endothelial NO production und induce production of reactive oxygen species (ROS) resulting in VSMC contraction. ADMA = asymmetric dimethylarginine, SDMA = symmetric dimethylarginine, AGE = advanced glycation endproducts, TXA_2 = Thromboxan A_2, PG = prostaglandins, AngII = angiotensin II, TGFβ = transforming growth factor β, TNFα = tumour necrosis factor α.

Abnormal endothelial secretory function

CKD is characterized by abnormal endothelial activation. Several substances circulating in the plasma of patients with impaired kidney function activate the transcription of distinct pro-inflammatory genes acting on endothelial transcription factors like NF-κB. The release of pro-coagulatory proteins (e.g. tissue factor) and activation of platelets induce a prothrombotic state. Vice versa, activated platelets secrete cytokines (e.g. IL-1β, RANTES), which further promote endothelial activation. Activated endothelial cells also produce cytokines, which act in an autocrine and paracrine manner by stimulation and chemotaxis of circulating leukocytes. Endothelial cell adhesion molecules enable adhesion and transmigration of mononuclear cells and T lymphocytes resulting in endothelial inflammation (Endemann et al. 2004; Rocha et al. 2009). Finally, abnormal endothelial activation leads to progressive endothelial apoptosis and damage with shedding of endothelial micro-particles and detachment of endothelial cells (Martinez et al. 2005).

3.2 Chronic kidney disease and cardiovascular risk

Chronic kidney disease (CKD) represents a major public health problem. Within the last years, the prevalence of CKD in western population steadily increased. Current data indicate a prevalence for CKD of approximately 14 % (2012).

CKD is associated with an enhanced morbidity and mortality due to cardiovascular events. The adjusted survival probability in patients with end-stage renal disease (ESRD) 60 months after initiation of renal replacement therapy is only 30 % and the risk of ESRD patients to die within the first year after initiation of renal replacement therapy is approximately 23 % (2012). With 41.6 %, cardiovascular disease represents the major cause of death in patients with ESRD. The mortality due to cardiovascular events is 10 to 100 fold increased in patients with ESRD as compared to age- and sex-matched control subjects without kidney disease (Foley et al. 1998; Go et al. 2004).

However, also patients in earlier stages of CKD exhibit an extremely elevated risk for cardiovascular events (Go, Chertow et al. 2004). Moreover, several studies identified a slightly reduced renal function or a microalbuminuria as strong cardiovascular risk factors (Foley, Parfrey et al. 1998; Garg et al. 2002; Garg et al. 2002; Arnlov et al. 2005; Shlipak et al. 2006; Menon et al. 2007; Van Biesen et al. 2007). Notably, most patients with CKD die before reaching ESRD because of cardiovascular events (Foley et al. 2005).

Traditional cardiovascular risk factors such as hypertension, diabetes, smoking, and dyslipidemia are highly prevalent in patients with CKD (Foley et al. 2005). However, CKD itself represents a cardiovascular risk and the presence of traditional risk factors alone may not be sufficient to explain the high cardiovascular morbidity and mortality in CKD patients (Muntner et al. 2005; Shlipak et al. 2005). Traditional and nontraditional cardiovascular risk factors in patients with CKD are summarized in the following table:

Traditional risk factors	**Nontraditional risk factors**	**Dialysis-related risk factors**
● Hypertension ● Dyslipidemia ● Diabetes ● Smoking ● Age	● Anemia ● Calcium-phosphate imbalance ● Proteinuria ● Inflammation and oxidative stress ● Coagulation ● Activation of renin-angiotensin-aldosteron system ● Hyperhomocysteinemia ● Left ventricular hypertrophy	● Bacteremia ● Chronic glycemia ● Extracellular fluid overload

Table 2 – Traditional and nontraditional cardiovascular risk factors in patients with CKD, adapted from (Rucker et al. 2009)

Moreover, the results from LDL-lowering studies in patients with CKD clearly document the presence of a different pathophysiology of cardiovascular disease in CKD patients as compared to those with a normal kidney function: While treatment with statins to reduce LDL cholesterol has convincingly been demonstrated to reduce cardiovascular risk in patients with normal kidney function at a high risk for cardiovascular events, similar studies in ESRD patients have not been proven beneficial (Baigent et al. 2011).

3.3 Vascular effects of High-Density Lipoprotein (HDL)

HDL plays a crucial role in the reverse cholesterol transport i.e. removing cholesterol from peripheral tissues.

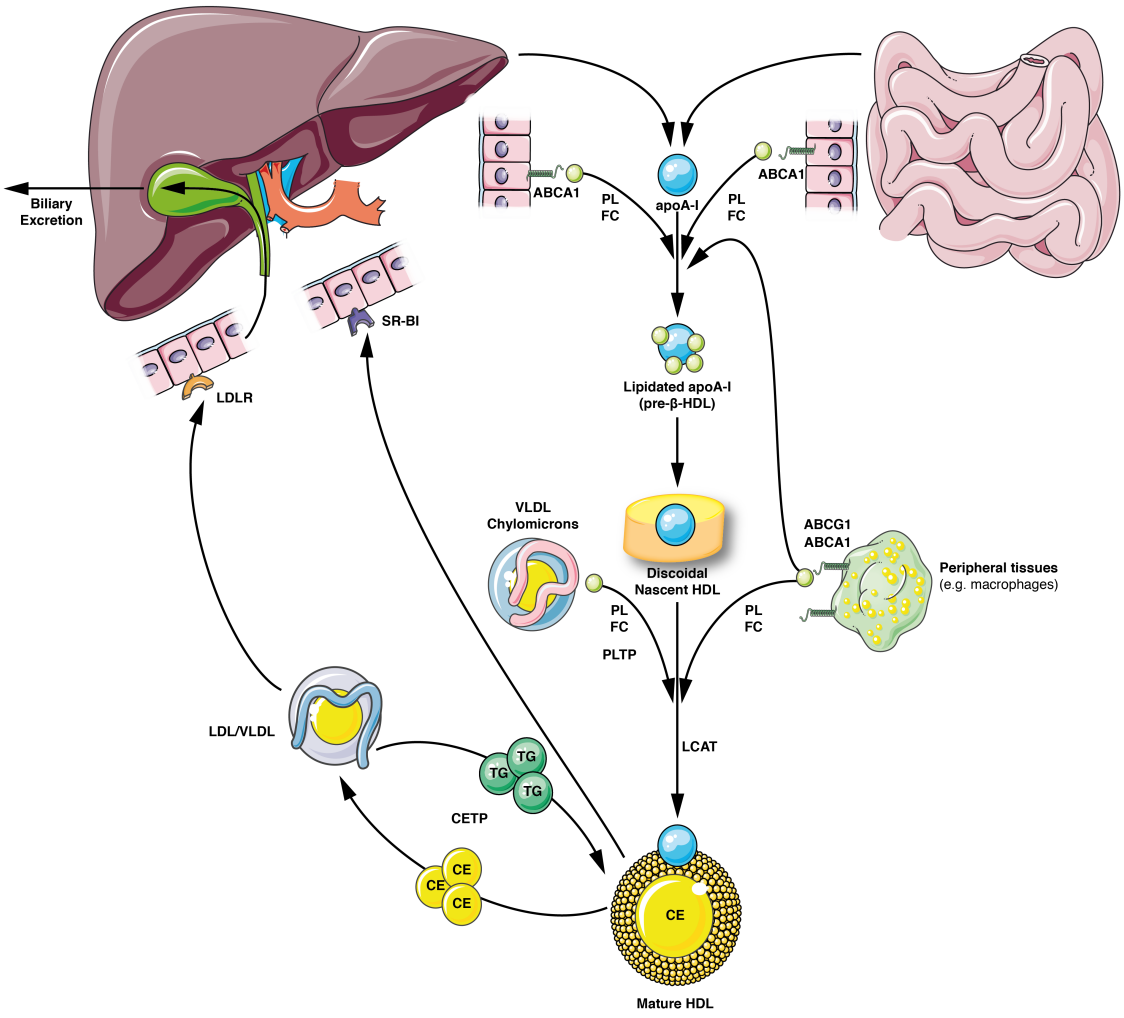

Figure 3 – HDL metabolism and reverse cholesterol transport (RCT). ABCA1, ATP binding cassette transporter A-1. ABCG-1, ATP binding cassette transporter G-1. PL, phospholipids. FC, free cholesterol. apoA-I, apolipoprotein A-I. VLDL, very low-density lipoprotein. PTLP, phospholipid transfer protein.

LCAT, lecithin:cholesterol acyltransferase. CE, cholesterol ester. CETP, cholesterol ester transfer protein. TG, triglycerides. LDLR, LDL receptor. SR-BI, scavenger receptor BI.

Mature HDL particles exhibit a complex structure consisting of a lipid and a protein moiety (apolipoprotein). Upon secretion by the liver and the intestine, apolipoprotein A-I (apoA-I) is immediately lipidated by accepting free cholesterol (FC) and phospholipids (PL) from liver, small intestine and peripheral tissues mediated by ATP binding cassette transporter A-1 (ABCA1). This results in the formation of the discoid nascent HDL. Subsequently, nascent HDL acquires further FC and PL from peripheral tissues as well as from hydrolysis of triglyceride-rich lipoproteins (e.g. VLDL) mediated by phospholipid transfer protein (PLTP), lipoprotein lipase.

The HDL-associated enzyme lecithin:cholesterol acyltransferase (LCAT) induces the esterification of free cholesterol to cholesterol ester (CE) accumulated in the nascent HDL particle. The increasing hydrophobicity evokes an accumulation of CE in the core of the HDL particle, which gives rise to the typical spherical structure of the mature HDL particle.

The removal of CE from HDL may occur via direct interactions between HDL and the hepatic scavenger receptor BI (SR-BI) or by the exchange from CE in the HDL fraction with triglycerides from apoB containing lipoproteins (VLDL, LDL), a process which is mediated by the lipid transporter cholesterol ester transfer protein (CETP). ApoB containing lipoproteins loaded with CE are then cleared from the circulation by the hepatic LDL receptor (LDLR).

Beside its role in the reverse cholesterol transport, HDL from healthy subjects exerts several potent vasoprotective effects mainly by targeting endothelial cells. HDL increases the endothelial NO bioavailability and reduces the production of reactive oxygen species (ROS) in endothelial cells. Additionally, HDL prevents endothelial pro-inflammatory and pro-coagulatory activation. Moreover, HDL reduces apoptosis of endothelial cells and stimulates endothelial repair mechanisms.

3.3.1 HDL and endothelial NO production

NO plays an important in the regulation of vascular integrity. In vascular endothelial cells, HDL from healthy subjects promotes the formation of NO by modulating the activity of endothelial NO synthase (eNOS), the endothelial NO producing enzyme. The pathways mediating endothelial NO production in response to HDL from healthy subjects are summarized in the following figure.

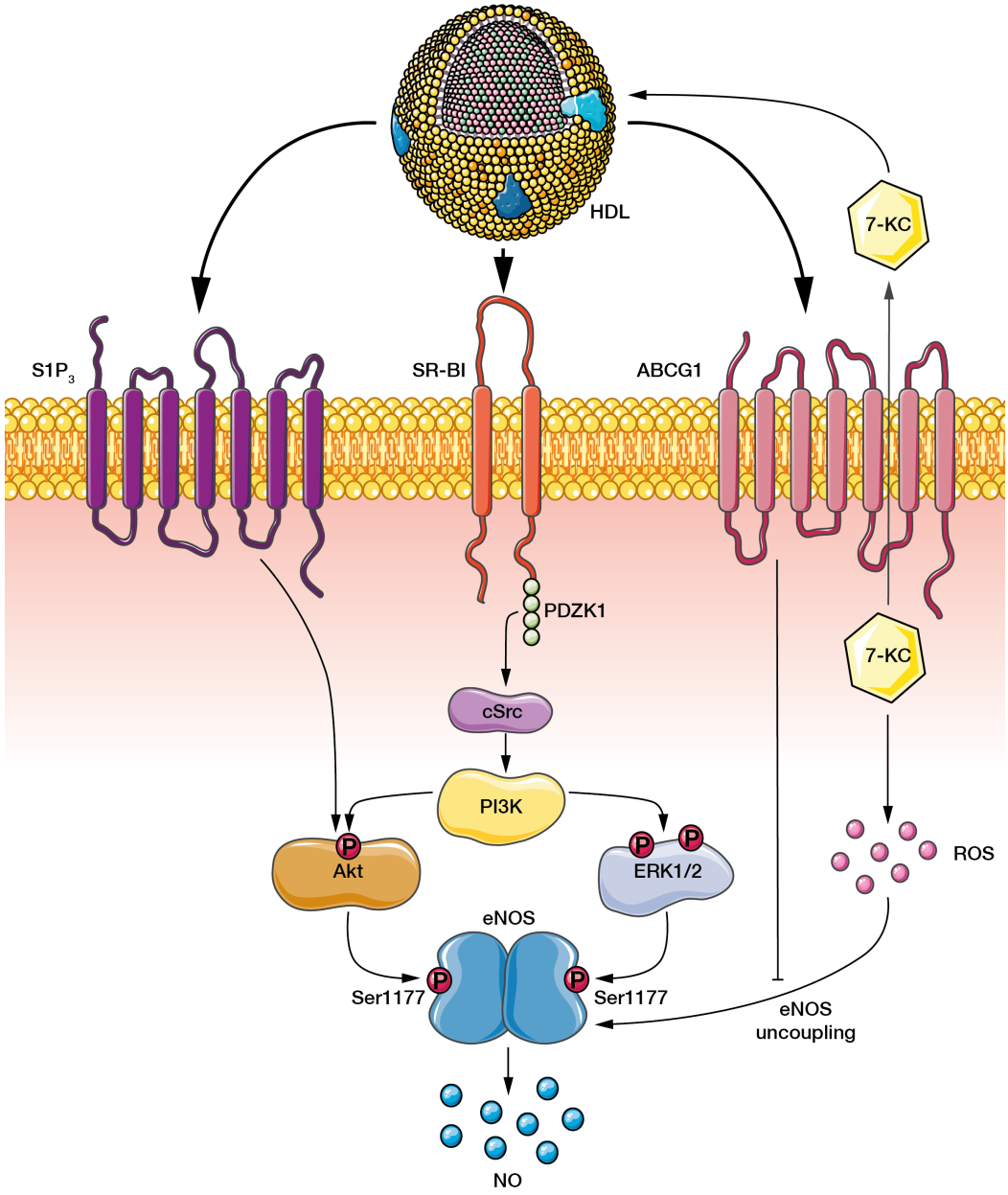

Figure 4 – Molecular pathways of HDL-mediated NO production in endothelial cells. $S1P_3$, sphingosine-1 phosphate receptor 3. SR-BI, scavenger receptor BI. 7-KC, 7-ketocholesterol. ABCG1, ATP binding cassette transporter G-1. PZDK1, multi PDZ domain-containing adaptor protein 1. PI3K, phosphoinositide 3-kinase. Akt, protein kinase B (PKB). ERK1/2, extracellular signal-regulated kinases 1/2. ROS, reactive oxygen species. eNOS, endothelial NO synthase. P, phosphorylation.

SR-BI was the first receptor on the surface of endothelial cells identified to mediate NO production in response to HDL (Yuhanna et al. 2001). Being colocalized with eNOS in endothelial caveolae, SR-BI induces the activation of phosphoinositide 3-kinase (PI3K) in endothelial cells. Subsequently, PI3K induces the phosphorylation Akt (protein kinase B, PKB) at Serine 473 residue, which then phosphorylates eNOS at Serine 1177 residue. Serine 1177 represents an eNOS-activating phosphorylation site, which has been identified as an

important regulatiry site of eNOS enzymatic activity (Dimmeler et al. 1999; Assanasen et al. 2005). Furthermore, it has been documented that activation of PI3K also induces phosphorylation of the mitogen-associated protein kinases (MAPK) extracellular signal-regulated kinases 1/2 (ERK1/2) in endothelial cells. Similarly, ERK1/2 phosphorylates eNOS at Serine 1177 residues and activates eNOS to produce NO (Mineo et al. 2003).

Moreover, sphingosine-1 phosphate, a major lipid constituent of HDL, also induces endothelial NO production via interaction with its sphingosine-1 phosphate receptor 3 ($S1P_3$). Thereby, interaction of HDL with the endothelial $S1P_3$ receptor activates eNOS via PI3K-dependent phosphorylation of Akt at Serine 473 residue and via direct PI-PLC (phosphatidylinositol-specific phospholipase C) dependent phosphorylation of eNOS at Serine 1177 residue (Nofer et al. 2004; Theilmeier et al. 2006).

Recently, the ATP binding cassette transporter ABCG-1, which plays an important role in the transport of phospholipids and free cholesterol from macrophages to HDL, was identified to be also involved in the regulation of HDL-mediated NO production in endothelial cells. ABCG-1 prevents endothelial dysfunction by preventing uncoupling of eNOS. Moreover, ABCG-1 mediates the export of 7-ketocholesterol (7-KC) from endothelial cells, which reduces endothelial ROS production and stabilizes eNOS conformation in order to maintain its enzymatic activity (Terasaka et al. 2008).

Notably, the relevance of these findings has been proven in hypercholesterolemic patients, in whom infusion of reconstituted HDL (rHDL) improved endothelial function in a NO-dependent manner as determined by flow-mediated vasodilation (FMD) (Spieker et al. 2002).

3.3.2 Impact of HDL on other endothelial-protective properties

Besides its important impact on NO bioavailability, HDL from healthy subjects exerts additional beneficial effects on the endothelium promoting vascular integrity.

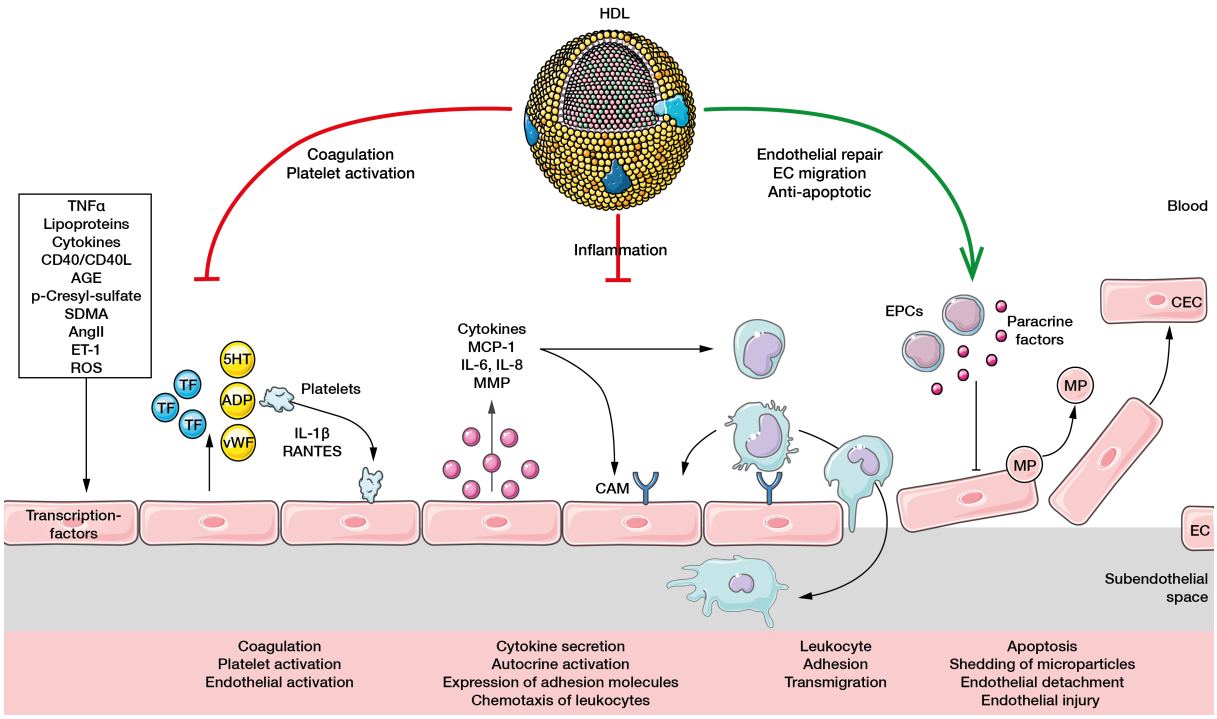

Figure 5 – Effects of HDL from healthy subjects on endothelial procoagulatory and proinflammatory activation as well as on endothelial repair. TNFα, tumor necrosis factor α. SDMA, symmetric dimethylarginine. AngII, angiotensin II. ET-1, endothelin 1. ROS, reactive oxygen species. TF, tissue factor, 5HT, serotinin. vWF, von Willebrand factor. IL-1β, interleukin 1β. RANTES, regulated and normal T cell expressed and secreted. MCP-1, monocytes chemotactic protein-1. MMP, matrix metalloprotease. CAM, cell adhesion molecule. EC, endothelial cell. EPC, endothelial progenitor cell. MP, microparticle. CEC, circulating endothelial cell.

Several substances circulating in blood of patients with CKD such as proinflammatory cytokines, uremic toxins, methylarginines, angiotensin II, endothelin-1 and reactive oxygen species lead to an abnormal activation of endothelial cells. In response to these stimuli, vascular endothelial cells produce a variety of mediators, which convey the endothelium into a procoagulatory, proinflammatory and proapoptotic state.

Regulation of endothelial procoagulatory activation

Abnormally activated endothelial cells secrete several procoagulatory factors, which on one hand induce the activation of plasmatic coagulation pathways and on the other and the activation of platelets. In contrast to these factors, HDL from healthy subjects exerts antithrombotic effects on endothelial cells and platelets. HDL reduces abnormal endothelial activation by reducing the expression of tissue factor (TF) and P/E-selectin on endothelial cells. In addition, HDL diminishes the generation of thrombin via upregulation of the anti-coagulatory factors activated protein C (APC), protein S as well as thrombomodulin. Moreover, HDL inhibits the activation of platelets by decreasing the secretion of platelet activating factor (PAF) and thromboxane A_2 (Mineo et al. 2006).

Importantly, the antithrombotic activity of HDL is broadly modulated by its effect on endothelial NO production. NO is known to preserve blood flow, to reduce endothelial activation and to inhibit platelet activation. Thus, NO generation in response to HDL does not only affect the vascular tone, but also HDL's antithrombotic properties.

Regulation of endothelial proinflammatory activation

The expression of adhesion molecules and the secretion of proinflammatory and chemotactic cytokines by the activated endothelium represents a prerequisite for endothelial adhesion and transmigration of mononuclear cells into the subendothelial layer and, thereby, for the formation atherosclerotic lesions and plaques. It has been previously shown that HDL from healthy subjects reduces the expression of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) on endothelial cells (Cockerill et al. 1995; Patel et al. 2010). Moreover, HDL attenuates the expression of CD11b on mononuclear cells and consequently reduces their adhesion to activated endothelial cells (Murphy et al. 2008). Notably, the anti-inflammatory properties of HDL are at least partially mediated by eNOS and the interaction between HDL and SR-BI as well as S1P receptors (Kimura et al. 2006).

Regulation of endothelial apoptosis

Apoptosis, the programmed cell death of endothelial cells is a crucial part of atherogenesis. A broad variety of stimuli (such as TNFα, oxLDL, IL-1β, ROS), circulating in the blood of patients with cardiovascular risk factors, may induce endothelial cells to undergo apoptosis (Dimmeler et al. 2002). It has been documented that HDL feature an intrinsic anti-apoptotic activity. In response to several stimuli, the constituents of HDL, apo-AI as well as spingosine-1 phosphate reduce endothelial caspase-3 activity, maintain the mitochondrial membrane potential and reduce the formation of ROS by the mitochondrial respiratory chain (Suc et al. 1997; Sugano et al. 2000; Nofer et al. 2001; Kimura et al. 2003).

HDL and endothelial repair

Several repair mechanisms may counteract the formation of endothelial lesions. Proliferation and migration of endothelial cells into de-endothelialized areas play an important role in preventing the formation of atherosclerotic lesions (Niimi et al. 1994). Moreover, despite an ongoing controversy about their phenotypic and ontogenetic definition, endothelial progenitor cells (EPCs) have been documented under several conditions to promote the repair of endothelial lesions presumably by paracrine effects (Bahlmann et al. 2010; Fadini et al.

2012). Clinical studies documented an associaton between the number of circulating EPCs and cardiovascular outcome (Schmidt-Lucke et al. 2005; Werner et al. 2005).
HDL induces both migration and proliferation of endothelial cells (Tauber et al. 1981; Murugesan et al. 1994; Tamagaki et al. 1996). Interestingly, these effects of HDL are also mediated via SR-BI and S1P (Seetharam et al. 2006). Moreover, HDL stimulates phosphorylation of Akt in human peripheral blood mononuclear cells (PBMC), their differentiation into EPCs as well as their migratory and tube formation activity in an SR-BI, PI3K and eNOS dependent manner (Tso et al. 2006; Sumi et al. 2007; Feng et al. 2009). In vivo, HDL increases capillary density and blood flow recovery in a hindlimb ischemia model. Furthermore, re-endothelialized after perivascular carotid injury was reduced in *apoA-I*$^{-/-}$ mice as compared to *apoA-I*$^{+/+}$ mice. In these experiments, the effect of HDL on endothelial repair was blunted in SR-BI deficient mice (Seetharam, Mineo et al. 2006).
Taken together these findings clearly indicate that HDL from healthy subjects not only protects endothelial integrity but also potently promotes endothelial repair mechanisms.

3.4 Aim of the present project

Recent evidence suggests the vascular effects of HDL can be heterogeneous. It has been demonstrated by our group that HDL can loose its vasoprotective under several disease conditions such as coronary artery disease, diabetes and antiphospholipid syndrome (Charakida et al. 2009; Sorrentino et al. 2010; Besler et al. 2011).
These observations led to the following hypothesis:

CKD alters the endothelial-protective properties of HDL and may therefore contribute to the high burden of cardiovascular disease in these patients.

Therefore, the aim of the present project was to examine the effect of HDL from CKD patients as a group of patients with an extraordinary high cardiovascular risk on distinct known endothelial functions of HDL.

4 Material and Methods

4.1 Animals

Wildtype (WT; C57BL/6J), *Tlr2*$^{-/-}$ (B6.129-Tlr2tm1Kir/J), *Tlr4*$^{-/-}$ (B6.B10ScN-Tlr4lps-del/JthJ), eNOS$^{-/-}$ (B6.129P2-Nos3tm1Unc/J) mice were obtained from The Jackson Laboratory, *Tlr1*$^{-/-}$ and *Tlr6*$^{-/-}$ mice from OrientalBioService, and CD1(nu/nu) from Charles River. Mice were housed and maintained in the Saarland University Hospital Animal Facility. All animal studies were approved by the animal ethics committee of the Saarland University and the University of Zurich, respectively.

4.2 ESR spectroscopy analysis of nitric oxide (NO) production in HAEC and BAEC

NO production was measured by ESR spectroscopy analysis with the use of the spin-trap colloid $Fe(DETC)_2$ as described previously (Kleschyov et al. 2000; Sorrentino, Besler et al. 2010). Cells were stimulated with the isolated lipoproteins or Pam3CSK4, respectively, for 1 hr.

4.3 ESR spectroscopy analysis of superoxide production in HAEC

Endothelial superoxide was measured using ESR spectroscopy and the spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) as described previously (Landmesser et al. 2002; Sorrentino, Besler et al. 2010). Cells were stimulated with the isolated lipoproteins or Pam3CSK4, respectively, for 1 hr.

4.4 Blood pressure measurements in mice

Systolic blood pressures were measured by a computerized tail-cuff system (Visitech Systems) as described previously (Landmesser, Cai et al. 2002). On each day of blood pressure determination, 20 measurements were obtained and averaged for each mouse.

4.5 Bone-marrow transplantation

Bone-marrow transplantation experiments were performed as described previously (Kania et al. 2009). In brief, 6-8 weeks old wildtype (WT) or TLR2$^{-/-}$ mice were lethally irradiated with 2 x 6.5 Gy using Gammatron (Co-60) system, and reconstituted with 2x10^{7} donor bone-marrow cells from WT or TLR2$^{-/-}$ mice, respectively. After reconstitution all mice received prophylactic antibiotics in the drinking water and were housed in a specific pathogen-free environment for 6 weeks.

4.6 Clinical study design and participants

The clinical studies enrolled adults and children with chronic kidney dysfunction as well as respective healthy controls (**Tables S1-S2**). The studies were conducted with approval of the local ethics committees (Ethikkommission der Ärztekammer Saarland, Germany, Ethikvotum 06/10; Research Ethics Committee, Great Ormond Street Hospital, London, UK) and informed consent was obtained from all participants or their legal guardians, respectively.

4.7 Lipoprotein isolation

Lipoproteins were isolated from fresh, fasting plasma by density gradient ultracentrifugation (HDL: density 1.063 to 1.21 g/cm^3, LDL: density 1.006-1.063 g/cm^3) as described previously (Havel et al. 1955; Cavelier et al. 2006; Rohrer et al. 2006). Potassium bromide was used to adjust the density. Lipoprotein concentrations used in the present study were based on protein content, which was determined by Bradford assay. Purity of each lipoprotein preparation was assessed by SDS-PAGE and subsequent Coomassie Blue staining of the gel. Lipid-free human plasma Apo-A1 was further purified from delipidated HDL as described previously (Brown et al. 1969).

4.8 Preparation of reconstituted HDL (rHDL)

Reconstituted HDL (rHDL) comprising apoA1, POPC, and cholesterol was prepared as described previously in detail by the sodium cholate dialysis method using an apoA-I/POPC/cholesterol molar ratio of 1:100:10 (Oram 2003).

4.9 Culture of endothelial cells

Human aortic endothelial cells (HAEC) were obtained from Clonetics® and cultured in Endothelial Cell Growth Medium-2 (Clonetics®, Lonza, Verviers, Belgium) supplemented with 10 % Fetal Bovine Serum (FBS, Gibco, Invitrogen, Basel, Switzerland). Bovine aortic endothelial cells (BAEC) were cultured as described previously (Ohnsorg et al. 2011). Before the experiments, cells were starved in Endothelial Basal Medium (Lonza, Verviers, Belgium) with 0.5 % FBS overnight. Cells were used within passage 4 to 6.

4.10 Isolation and culture of murine bone-marrow derived macrophages

Murine bone-marrow cells were isolated from the femora of WT or TLR-/- animals, by flushing with sterile PBS and filtering through 40 μm cell strainers (BD Falcon). Cells were grown in RPMI1640 supplemented with 10 % FBS, penicillin/streptomycin, MEM non-essential amino acids, sodium pyruvate, 2-mercaptoethanol (Gibco, Invitrogen) and 50 ng/ml M-CSF (Peprotech) for 6 days.

4.11 Determination of methylarginines using HPLC-ESI-MS/MS

EDTA-plasma or lipoprotein solution (50 μl) was supplemented with 20 μl internal standards solution (460 μmol/L $^{13}C_6$-Arg and 45 μmol/L D7-ADMA both from Cambridge Isotope Laboratories, Andover, USA). To precipitate the proteins 200 μl of mixture methanol/acetonitrile / 0.1 M aqueous zinc sulphate (80% + 10% + 10%, v/v/v) (Sigma) was added. After centrifugation at 16,000 x g for 10 min at 10°C, the supernatant was collected and dried under nitrogen at 25°C. The dried sample was derivatised with 100 μl butanol solution containing 3 M HCl (Regis technologies, Socochim Lausanne) at 65 °C for 20 min. The derivatised samples were dissolved in 100 μL aqueous 0.2% trifluoroacetic acid (v/v) solution.

For the HPLC separation a Rheos 2000 pump with a degasser Rheos DSO-LC (Flux Instruments, Basel, Switzerland) and Betasil Phenyl-Hexyl (Symmetry, 3 µm, 3 × 100 mm) (Thermo Fisher Scientific) was used. The flow rate was 300 μL/min with a gradient elution of solution A (0.2% aqueous trifluoroacetic acid, v/v) and solution B (acetonitril). The percentage of organic modifier (B) was changed linearly as follows: 1 min, 5%; 5 min, 50%; 2.5 min isocratic, 50%; 2.5 min, 5%. The injection volume was 10 μL and the total analysis run time was 10 min.

Mass spectrometric analyses were performed using a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer equipped with an electrospray ion source (ESI) operating in

positive mode. Observed multiple-reaction monitoring (MRM) transitions were: m/z 259 > 228 for SDMA, m/z 259 > 214 for ADMA, and m/z 266 > 77 for its IS (ADMA-D7). Data collection and analysis were done with Thermo Xcalibur software package, revision 1.2.

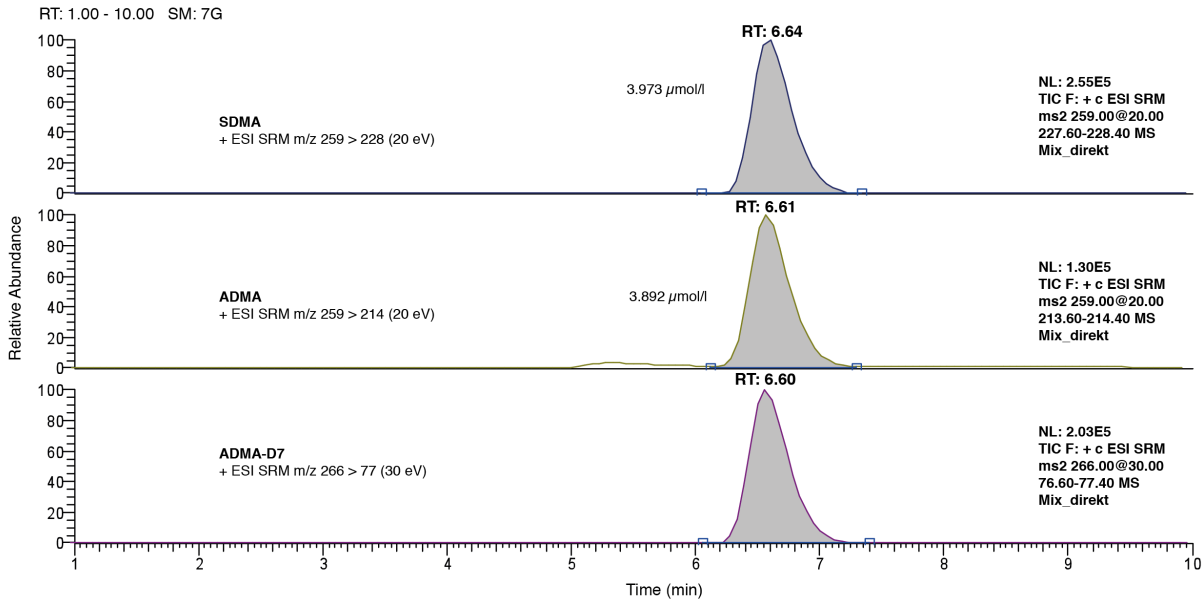

Figure 6 - Representative mass chromatograms obtained for the standard mixture of SDMA, ADMA and ADMA-D_7. Following transitions were used for quantification: *m/z* 259 > 227 (SDMA), *m/z* 259 > 214 (ADMA) and *m/z* 266 > 77 (ADMA-D_7). The concentrations in this mixture were 4 µmol/L for SDMA, ADMA and ADMA-D_7, respectively. We obtained the following concentrations: SDMA 3.973 µmol/L, ADMA 3.892 µmol/L.

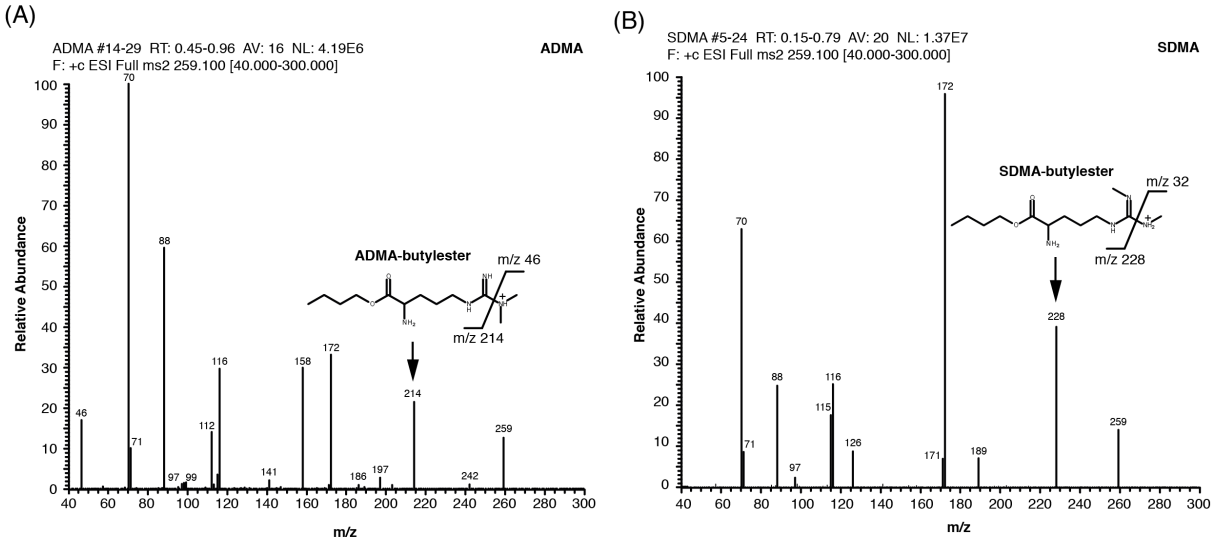

Figure 7 - ESI-MS/MS product ion spectra pattern obtained for (A) ADMA and (B) SDMA. Specific fragments used for quantifications are marked with arrows (ADMA 214 m/z, SDMA 228 m/z).

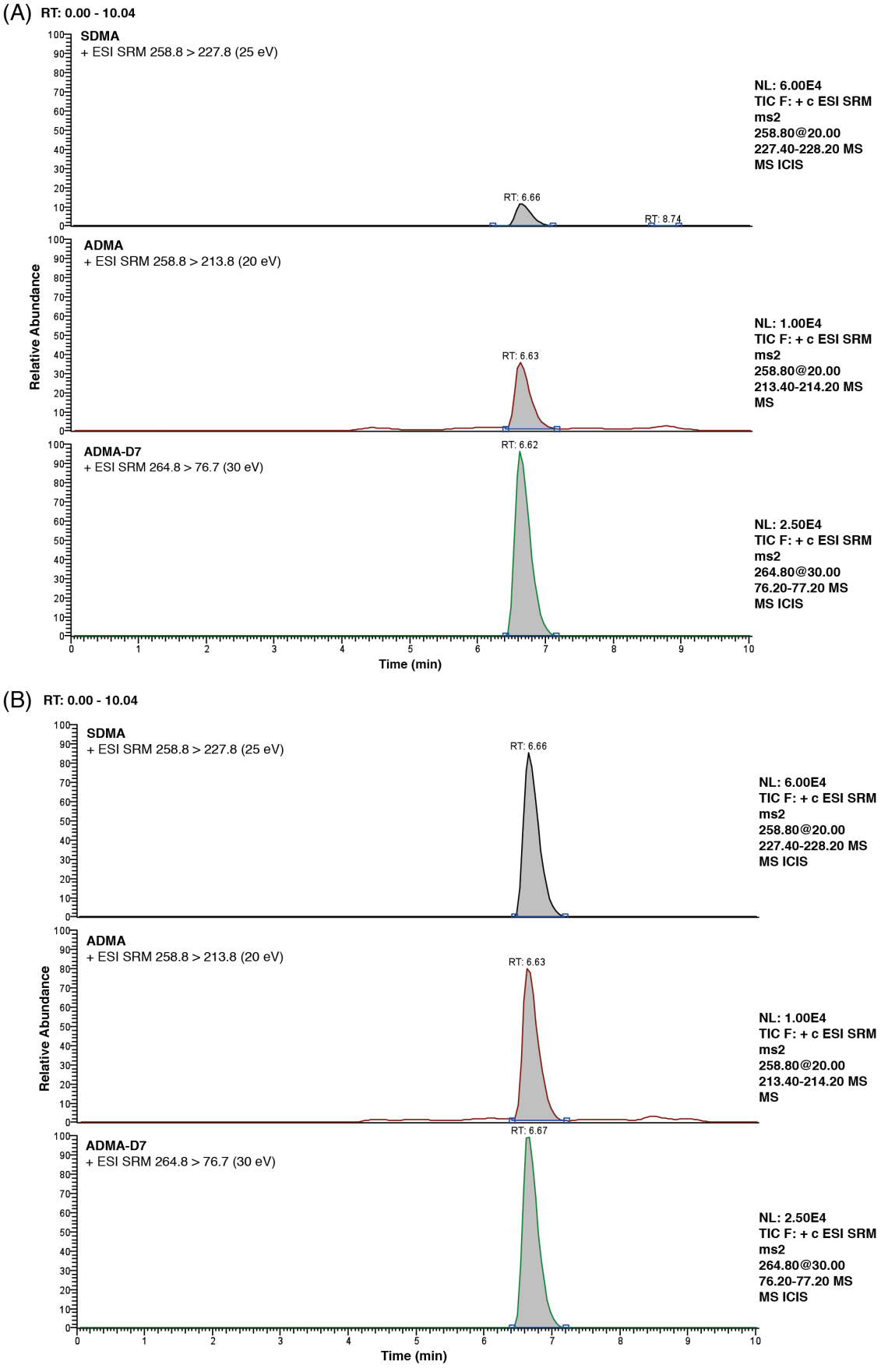

Figure 8 - Representative mass chromatograms obtained from plasma (A) of a healthy subject and (B) a patient with kidney dysfunction.

4.12 Supplementation of lipoproteins with methylarginines

HDL or LDL were incubated with the indicated concentrations of SDMA or ADMA overnight at 4 °C on a shaker. Lipoproteins were used after dialysis against Krebs-Henseleit buffer.

4.13 Endothelial cell migration assay

In vitro scratch assay was used to assess endothelial cell migration as described previously (Liang et al. 2007). HAEC were grown until confluency in 24 well plates in EGM-2 medium supplemented with 10 % FCS. For the assay, medium was changed to EBM containing 0.5 % FCS and 50 µg/ml HDL was added. Two parallel linear scratches were performed with a 200 µl pipette tip and pictures were taken at 4 different positions by phase contrast microscopy immediately after scraping and 24 hours later at the same positions. Newly closed distance was calculated by subtracting the width of the scratch after 24 hrs from the width at time point 0. Each experiment was performed in triplicates and mean was calculated.

4.14 Flow cytometric detection of TLR expression

TLR expression was assessed by flow-cytometry using appropriate antibodies targeting TLR-2 (clone: 11G7), TLR-4 (clone: HTA125) (BD Pharmingen and Immunokontact), TLR-1 (clone: H2G2) and TLR-6 (both from Invivogen).

4.15 Carotid injury model

Female CD1nu/nu nude mice, aged 7 to 10 weeks, were anaesthetized with isoflurane. Carotid artery electric injury was performed as described previously (Carmeliet et al. 1997; Sorrentino et al. 2007; Sorrentino, Besler et al. 2010). In brief, the left common carotid artery was injured with a bipolar microregulator (ICC50, ERBE-Elektromedizin GmbH, Tuebingen, Germany). An electric current of 2 W was applied for 2 seconds to each millimeter of carotid artery over a total length of exactly 4 mm with the use of a size marker parallel to the carotid artery. 15 mg/kg of HDL were injected in volume of 250 µl 3 hours after carotid injury via tail vein injection with a 30-gauge needle. Three days after carotid injury,endothelial regeneration was evaluated by staining denuded areas with 50 µl of solution containing 5% Evans blue dye via tail vein injection. The re-endothelialized area was calculated as difference between the blue-stained area and the injured area by computer-assisted morphometric analysis. This model has been shown to allow accurate quantification of reendothelialization (Carmeliet,

Moons et al. 1997). HDL from each CKD/healthy subject were injected into 2 nude mice, and mean values of re-endothelialized area were used for analysis.

4.16 Endothelial mononuclear cell adhesion assay

HAECs were seeded in 24 well plates and grown until confluency. After serum withdrawal overnight, they were stimulated with TNFα (5 ng/ml, 4 hrs) and HDL (50 µg/ml, 3 hrs). Peripheral blood mononuclear cells (PB-MNCs) were separated from peripheral whole blood of a healthy human volunteer by Ficoll density gradient centrifugation as described previously (Krankel et al. 2008). PB-MNCs were labelled with DiI according to the manufacturer's protocol (VybrantTM Cell- Labeling Solutions, Molecular Probes, Invitrogen). Afterwards, $1x10^6$/well of the labelled MNC were added to the HAECs. After a 4 hrs incubation period, non-adherent PB-MNCs were carefully removed by washing with phosphate buffered saline (PBS). The integrity of the endothelial monolayer was confirmed by staining with 4',6-diamidino-2-phenylindole (DAPI, Vectashield, Reactolab, Servion, Switzerland). Adherent DiI-labelled PB-MNCs were counted in 4 randomly selected high-power fields using a fluorescent microscope (DM-IRB, Leica) connected to a digital imaging system (Spot-RT; Diagnostic Instrument/Visitron Systems).

4.17 Generation of HEK-293 cells expressing hSR-BI

The hSR-BI cDNA was inserted into pcDNA3 vector and transfected into HEK-293 cells using lipofectamin 2000. As control pcDNA3 vector without insert was transfected into HEK-293 cells. Transfected HEK-293 cells were selected in DMEM containing 10% FBS, 2 mM glutamine, G418 400 μg/ml, 50 units/ml penicillin and 50 µg/ml streptomycin. Binding studies were performed as described for endothelial cells.

4.18 Endothelial cell binding and cell association assays

Endothelial binding (i.e. binding to the cell surface) and association (i.e. binding to the cell surface and internalization) assays using ^{125}I-HDL were performed as described previously (Ohnsorg, Rohrer et al. 2011). Briefly, bovine aortic endothelial cells (BAEC) were incubated with ^{125}I-HDL without (total) or with (unspecific) a 40-fold excess of unlabeled HDL as competitor for 2 hrs at 4 °C or for 1 hr at 37 °C to quantify binding or cell association, respectively. After incubation, the amounts of cell bound radioactivity were determined using a Perkin Elmer γ-counter and the protein content was analyzed as described previously

(Rohrer, Cavelier et al. 2006). Specific binding/cell association was calculated by subtracting the values of nonspecific binding/cell association from those of total binding/cell association. All experiments were performed at least in triplicates.

4.19 Western Blot techniques

Protein expression was determined by Western blot analysis. Cells were lysed in lysis buffer containing 50 mmol/l Tris pH 7.5, 150 mmol/l NaCl, 1 mmol/l EDTA, 0.5 % NP-40 supplemented with protease and phosphatase inhibitors (10 µg/ml Aprotinin, 10 µg/ml Leupeptin, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF). Protein concentration was determined by the Bradford assay. 30 µg of protein were loaded per lane, resolved by 10 % SDS-PAGE, transferred to a PVDF membrane (Millipore, Billerica, MA, USA) by semidry transfer. The following antibodies and dilutions were used: Goat anti-human VCAM-1 (R&D systems, Abingdon, UK, polyclonal) 1:2,000, Mouse anti-human eNOS (pT495) (BD transduction laboratories, clone: 31/eNOS(pT495) 1:1,000, Mouse anti-human eNOS (pS1177) (BD transduction laboratories, clone: 19/eNOS/S1177) 1:1,000, Mouse anti-human eNOS/NOS Type III (BD transduction laboratories, clone: e/eNOS/NOS Type III) 1:1,000, Rabbit anti-human Phospho-Akt (Ser473) (Cell signaling, clone: 193H12) 1:2,000, Rabbit anti-human Akt (pan) (Cell signaling, clone: 11E7) 1:2,000, Rabbit anti-human Phospho-SAPK/JNK (Thr183/Tyr185) (Cell signaling, clone: 81E11) 1:2,000, Rabbit anti-human SAPK/JNK (Cell signaling, clone: 56G8) 1:5,000, Mouse-anti human GAPDH (Millipore, clone: 6C5) 1:20,000 as loading control.

4.20 Fluorescent labeling of HDL using Atto-488

4 mg of HDL from healthy subjects have been diluted in PBS to achieve a final volume of 300 µl. Afterwards the HDL solution together with 35 µl sodium bicarbonate buffer (1.0 M, pH 9.5) was added to a tube containing 25 µl Atto-488-NHS (AttoTec, Germany) and incubated for 2 hrs at room temperature. After incubation, Atto-488-labeled HDL was purified using gel filtration.

4.21 Determination of the interaction between Atto-488-HDL and endothelial cells

Endothelial cells were transfected with a pZERO plasmid containing a dominant-negative form of the human TLR-2 (TLR2-ΔTIR-HA) or TLR-5 gene (TLR5-ΔTIR-HA) containing an

HA-tag by using Lipofectamine 2000 (Invitrogen, 2.5 μg DNA, 7.5 μl Lipofectamine in 250 μl Opti-MEM medium). Transfection efficiency was assessed by measuring TLR surface expression as well as by western blot using an anti-HA antibody (Invivogen). 48 hrs after transfection, cells were incubated with Atto-488-labeled HDL for 30 min at 37 °C. After several washing steps, cells were immediately analyzed for the presence of Atto-488-HDL using flow-cytometry.

4.22 TLR blocking experiments

To neutralize TLR-1 (clone: H2G2), TLR-2 (clone: B4H2), TLR-4 (clone: W7C11), TLR-6 (clone: C5C8), cells were incubated with the respective neutralizing antibodies (Invivogen) for 1 hr before stimulation with HDL or TLR agonists.

4.23 Cell-based TLR-2 activation assay

HEK293 cells co-transfected with the human TLR-2 and SEAP (secreted embryonic alkaline phosphatase) genes were purchased from Invivogen. In these cells, the SEAP gene is placed under control of the IFN-β promoter and fused to five NF-κB and AP-1 binding sites. Stimulation of TLR-2 activates NF-kB and AP-1, which then induce the production of SEAP. Notably, these cells express endogenous level of TLR-1 and TLR-6. Cells were cultured in DMEM medium containing 4.5 g/l glucose, 10 % FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml Normocin (Invivogen) and 2 mM L-glutamine. After overnight stimulation of the cells with the respective agents, SEAP in the cell culture medium was colorimetrically quantified using QUANTI-Blue detection medium (Invivogen) with a platereader at 635 nm.

4.24 ELISA

To determine the concentrations of the cytokines TNF, IL-1β, IL-6, IL-10 IL-12 p40 in cell culture supernatant, commercially available ELISA kits (BD OptEIA for mouse, eBioscience and Biolegend for human) were used according to the manufacturer's instructions.

4.25 Intracellular cytokine staining

Intracellular staining for the production of TNFα and IL-6 was performed as described previously (Schmidt et al. 2012). Stimulation was performed at 37 °C for 12 hrs (HAEC) or 6 hrs (human PBMCs).

4.26 Statistics

All data are expressed as mean ± SEM. Statistical comparisons were made by one-way analysis of variance or the nonparametric Kruskal Wallis test. P-value <0.05 was considered statistically significant. Post-hoc Tukey test or Bonferroni adjustment was performed for multiple comparisons. All analyses were performed with GraphPad Prism (Version 4.0, GraphPad Software, Inc.).

5 Results

5.1 Patient recruitment

For the isolation of HDL from serum samples, adult and children with different degrees of CKD as well as respective healthy control subjects were recruited. The clinical characteristics of the respective cohorts are provided in the following tables:

	Healthy Controls N=15	**CKD-II° N=15**	**CKD-III/IV° N=15**	**CKD-V° N=15**	***P***
Age (years)	60 ± 8	60 ± 11	67 ± 12	64 ± 11	0.153
BMI (kg/m^2)	26.7 ± 2.8	29.1 ± 3.9	30.1 ± 5.5	28.1 ± 5.5	0.177
Male (%)	55.6	57.9	53.3	66.7	0.902
Diabetes mellitus (%)	-	15.8	26.7	40.0	0.386
HbA1c (%)	5.8 ± 0.3	5.8 ± 0.6	6.4 ± 1.2	5.4 ± 0.6	0.018
CAD (%)	-	10.5	33.3	46.7	0.012
Hypertension (%)	-	94.7	93.3	93.3	0.000
MAP (mmHg)	101 ± 7	103 ± 10	96 ± 12	98 ± 13	0.157
CRP (mg/dl)	1.9 ± 1.6	3.3 ± 2.7	3.6 ± 3.3	6.3 ± 6.1	0.035
Total cholesterol (mg/dL)	191 ± 11	203 ± 47	185 ± 45	169 ± 57	0.116
Triglycerides (mg/dL)	122 ± 73	170 ± 143	174 ± 91	156 ± 72	0,646
LDL cholesterol (mg/dL)	118 ± 28	123 ± 41	107 ± 35	96 ± 27	0.046
HDL cholesterol (mg/dL)	53 ± 16	49 ± 13	45 ± 9	36 ± 7	0.001
Creatinine (mg/dl)	0.8 ± 0.1	1.0 ± 0.2	2.3 ± 0.8	8.3 ± 3.9	0.000
eGFR, MDRD (mL/min/1.73 m^2)	102 ± 14	71 ± 14	32 ± 20	7 ± 3	0.000
Urea (mg/dl)	37 ± 12	44 ± 13	100 ± 31	136 ± 52	0.000
Current Medication					
ACE Inhibitors (%)	-	42.1	20.0	26.7	0.019
AT1 blocker (%)	-	47.4	20.0	20.0	0.023
Beta Blockers (%)	-	68.4	73.3	73.3	0.001
Ca- Antagonists (%)	-	26.3	20.0	20.0	0.386
Diuretics (%)	-	73.7	93.3	60.0	0.000
Statin therapy (%)	-	52.6	80.0	40.0	0.000

Table 3 - Clinical characteristics of adult patients with CKD and respective healthy control subjects

	Healthy Controls N=10	**CKD-III/IV° N=13**	**CKD-V° N=9**	***P***
Age (years)	13 ± 4	13 ± 2	14 ± 4	0.700
BMI (kg/m^2)	22	22.9 ± 2.8	23.3 ± 4.6	0.460
Male (%)	48	64	60	0.880
Diabetes mellitus (%)	-	-	-	
CAD (%)	-	-	-	
Hypertension (%)	-	15	11	0.000
CRP (mg/dl)	0.8 ± 1.4	1.1 ± 1.0	4.3 ± 2.9	0.042
Total cholesterol (mg/dL)	120 ± 27	143 ± 73	159 ± 81	0.065
Triglycerides (mg/dL)	70 ± 149	88 ± 44	114 ± 105	0.880
LDL cholesterol (mg/dL)	50 ± 27	66 ± 35	77 ± 46	0.063
HDL cholesterol (mg/dL)	73 ± 19	81 ± 39	93 ± 85	0.072
eGFR (mL/min/1.73 m^2)	112 ± 18	23 ± 12	8 ± 13	0.000
Current Medication				
ACE Inhibitors (%)	-	7.7	-	
AT1 blocker (%)	-	-	-	
Beta Blockers (%)	-	-	-	
Ca- Antagonists (%)	-	7.7	11.1	
Diuretics (%)	-	-	-	
Statin therapy (%)	-	-	-	

Table 4 - Clinical characteristics of children patients with CKD and respective healthy control subjects

5.2 Effect of HDL from healthy subjects and patients with CKD on endothelial NO bioavailability and superoxide production

At first, the production of NO in HAEC after incubation with $HDL^{Healthy}$ as well as HDL^{CKD} was studied by using ESR spectroscopy. PBS treated HAEC were used as control.

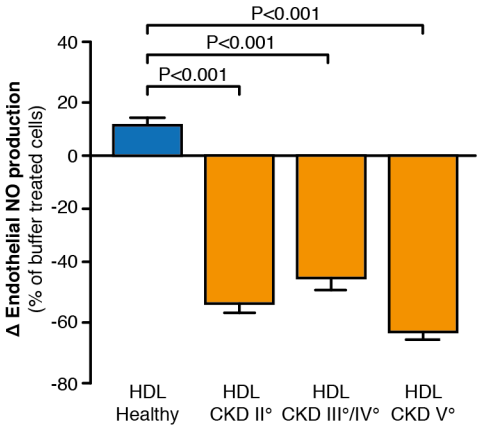

Figure 9 – Endothelial NO production in HAEC incubated for 60 min with HDL (50 µg/ml) determined by ESR spectroscopy (n=15 per group).

HDLHealthy stimulated endothelial NO production, whereas HDLCKD strongly inhibited endothelial NO production. Notably, this inhibitory effect on endothelial NO production was already present with HDL from patients with incipient CKD.

Since there is a frequent debate on HDL raising pharmaceutical therapies, next, endothelial NO production in response to increasing concentrations was quantified.

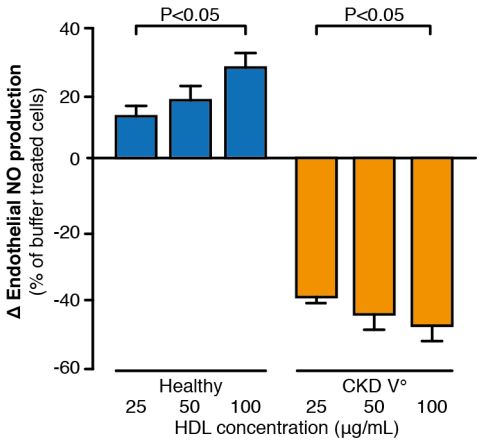

Figure 10 – Endothelial NO production in HAEC incubated for 60 min with increasing concentrations of HDL (25 µg/ml, 50 µg/ml, 100 µg/ml) determined by ESR spectroscopy (n=3-5 per group).

While increasing concentrations of $HDL^{Healthy}$ further stimulated endothelial NO production, we observed that higher concentrations of HDL^{CKD} led to a significantly stronger inhibition of endothelial NO production. Thus, we concluded the effects of $HDL^{Healthy}$ as well as HDL^{CKD} to be dose-dependent.

Since pathological conditions such as diabetes, hypertension and coronary artery disease, which are known to affects the functional properties of HDL, are frequently observed in adult

patients with CKD, we next analyzed the effect of HDL from children with CKD on endothelial NO production.

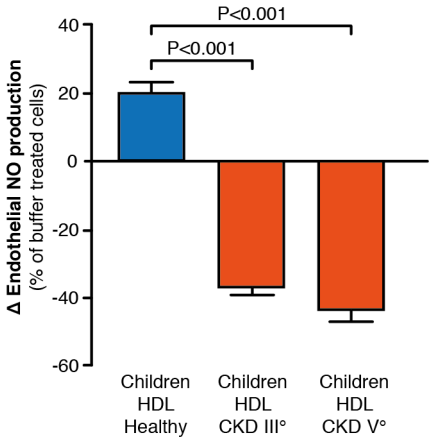

Figure 11 – Endothelial NO production in HAEC incubated for 60 min with HDL (50 µg/ml) from children with CKD and respective healthy control subjects (n=9-13 per group).

In this experiment, we observed that *HDL*^{CKD} from children also significantly reduced endothelial NO production. Therefore, we concluded that CKD per se and not concomitant diseases are responsible for the observed effects of *HDL*^{CKD} on endothelial NO bioavailability.

Since *HDL*^{CKD} substantially reduced endothelial NO bioavailability and since NO is known to be an important regulator of vascular tone, we next asked on whether *HDL*^{CKD} may also have an effect on arterial blood pressure *in vivo*. Therefore, we injected *HDL*^{Healthy} as well as *HDL*^{CKD} into mice and determined the response on systolic blood pressure.

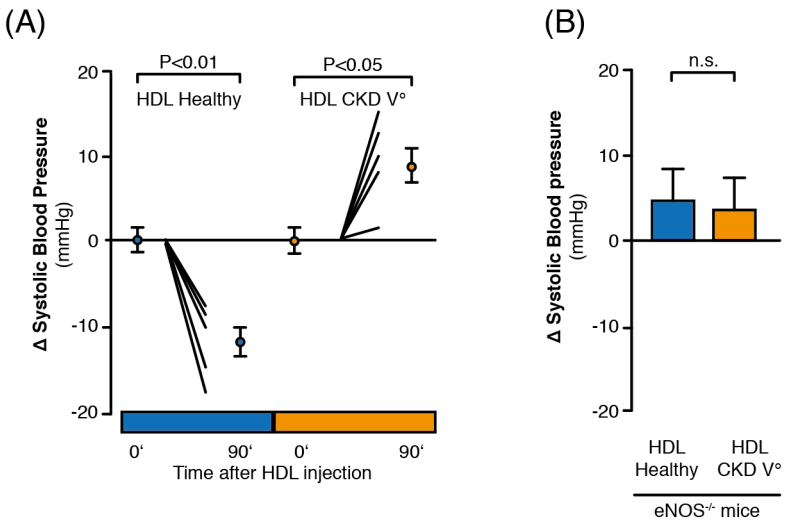

Figure 12 – Δ Systolic blood pressure in (A) wildtype and (B) eNOS-/- mice 90 min after injection of HDL (50 mg/kg body weight, n=4-6 per group).

Interestingly, in wildtype mice, HDL^{CKD} significantly increased the arterial blood pressure, whereas injection of $HDL^{Healthy}$ reduced the arterial blood pressure. To investigate as to whether these effects of HDL^{CKD} on arterial blood pressure are mediated by eNOS, we repeated these measurements in eNOS-/- mice. In these mice, $HDL^{Healthy}$ lost its ability to lower the arterial blood pressure indicating that the effects of HDL on arterial blood pressure might be mediated by eNOS.

To gain insights into the mechanism by which HDL^{CKD} may inhibit endothelial NO production, we next measured endothelial superoxide production in response to HDL. The superoxide radical is known to rapidly react with NO forming the peroxynitrite radical and, thereby, to reduce the NO bioavailability (Pacher et al. 2007).

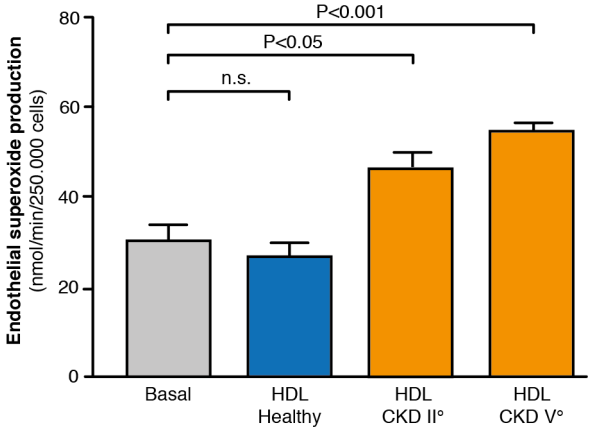

Figure 13 – Basal endothelial superoxide production in HAEC incubated with HDL (50 µg/ml) for 1 hr (n=6 per group).

Indeed, incubation of HAEC with HDL^{CKD} significantly promoted basal superoxide production, while HDLHealthy did not affect production of superoxide radicals in HAEC.

5.3 Effect of methylarginines on the endothelial properties of HDL

We hypothesized, that endogenous NO inhibitors such as asymmetric dimethylarginine (ADMA) or symmetric dimethylarginine (SDMA), which are known to accumulate in the serum from patients with CKD, may associate with HDL^{CKD} and alter its vasoprotective properties. Therefore, we established a HPLC MS/MS approach to determine the levels of ADMA and SDMA in the HDL fraction.

At first, we confirmed that the concentrations of both ADMA and SDMA are elevated in the serum from patients with CKD.

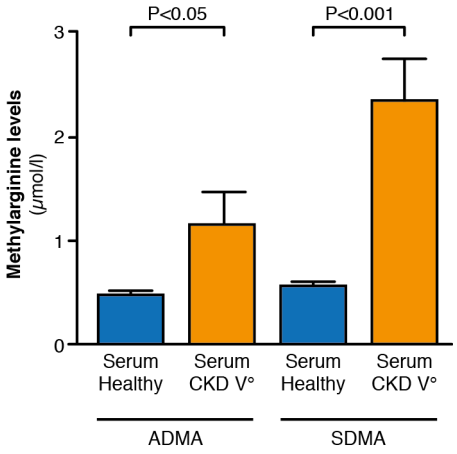

Figure 14 – Concentrations of ADMA and SDMA in the serum from patients with CKD and healthy subjects determined by HPLC/ESI-MS/MS (n=10 per group).

Next we determined the concentrations of ADMA and SDMA in the HDL fractions from patients with CKD as well as healthy control subjects.

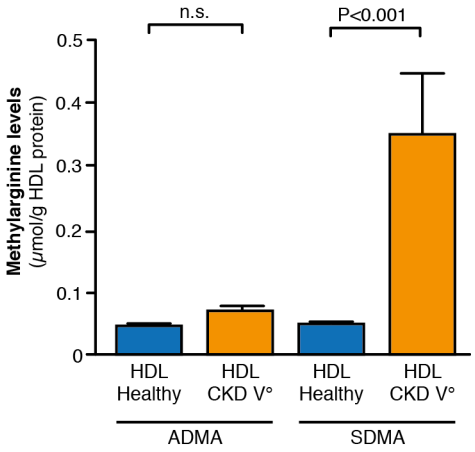

Figure 15 – Concentrations of ADMA and SDMA in the HDL fraction from patients with CKD and healthy subjects determined by HPLC/ESI-MS/MS (n=10 per group).

We could not detect any ADMA in the HDL fractions from healthy subjects and patients with CKD. However, we found SDMA to be significantly enriched in HDL^{CKD} but not in $HDL^{Healthy}$. Whereas ADMA is known to inhibit endothelial NO production, the effect of SDMA on endothelial NO bioavailability has not been examined. Therefore, we supplemented HDL and LDL from healthy subjects with SDMA or ADMA and determined the effect of the modified lipoproteins on endothelial NO production.

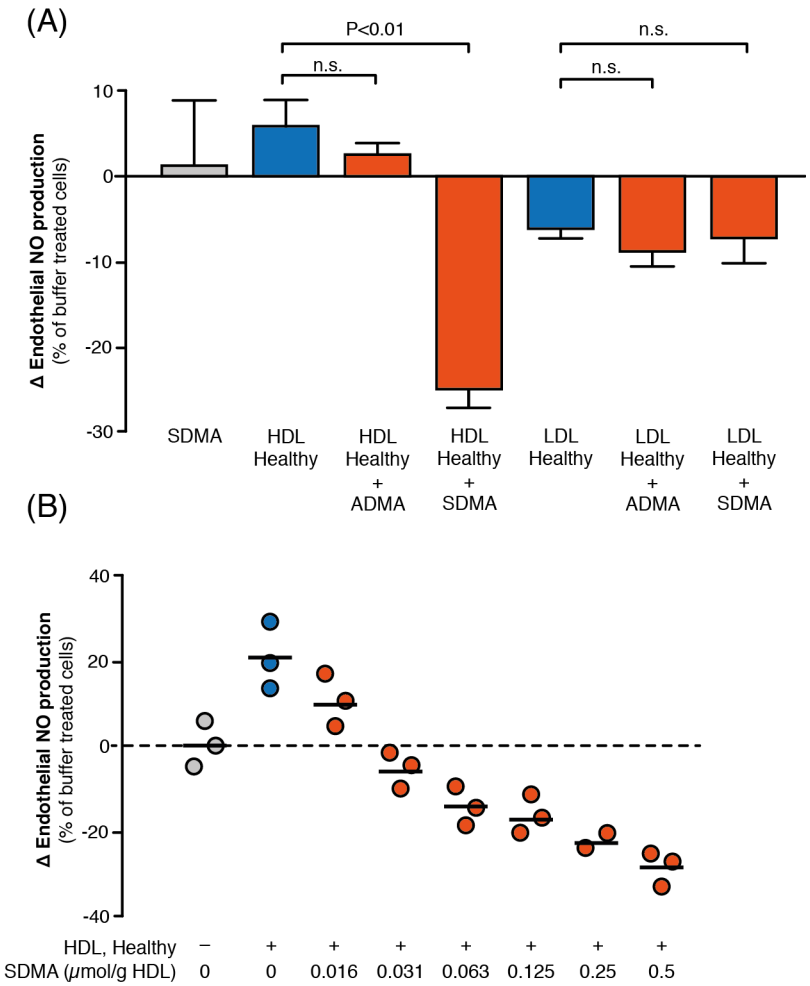

Figure 16 – (A) Effect of SDMA alone (4 μM), healthy HDL (50 μg/ml) or LDL (100 μg/ml) supplemented with or without SDMA or ADMA (4 μM equivalent to 0.5 μmol/g lipoprotein) on endothelial NO production (n=3-6 per group). (B) Effect of $HDL^{Healthy}$ supplemented with different concentrations of SDMA (0.5 μmol/g HDL protein) on endothelial NO production (n=3 per group).

We observed that *$HDL^{Healthy}$* supplemented with SDMA but not ADMA inhibited endothelial NO production. Notably, supplementation of LDL with SDMA or ADMA did not alter the effect of LDL on endothelial NO production. Moreover, SDMA alone did also not significantly change endothelial NO production. In dose-response experiments, SDMA concentrations, comparable to those measured in the HDL fraction of CKD patients, significantly reduced the production of NO in endothelial cells. These results indicate that SDMA may associate to HDL resulting in an inhibition of endothelial NO production.

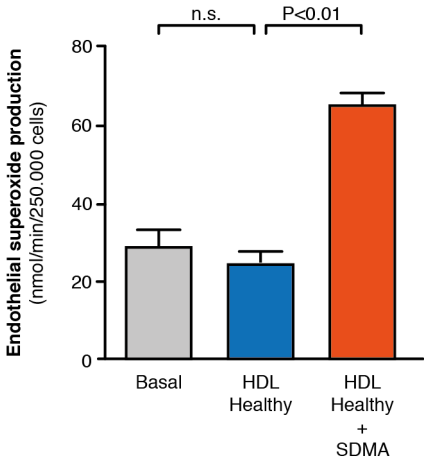

Figure 17 - Effect of HDL (50 µg/ml, 1 hr) supplemented with SDMA (0.5 µmol/g HDL protein) on endothelial superoxide production (n=6 per group).

Furthermore, we measured endothelial superoxide production in response to HDL supplemented with SDMA (HDL^{SDMA}). Similarly to HDL^{CKD}, we observed that HDL^{SDMA} stimulated basal endothelial superoxide production.

Furthermore, we supplemented reconstituted HDL (rHDL) consisting of Apo-A1:POPC:cholesterol in a molar ratio of 1:100:10 with and without SDMA and measured its effect on endothelial cell NO production. Supplementation of rHDL with SDMA reduced endothelial NO production. Moreover, after supplementation with SDMA, apolipoprotein A1 (Apo-A1) inhibited endothelial NO production, whereas Apo-A1 without SDMA did not significantly affect endothelial NO production. This indicates that SDMA may associate with Apo-A1, the major apolipoprotein of HDL.

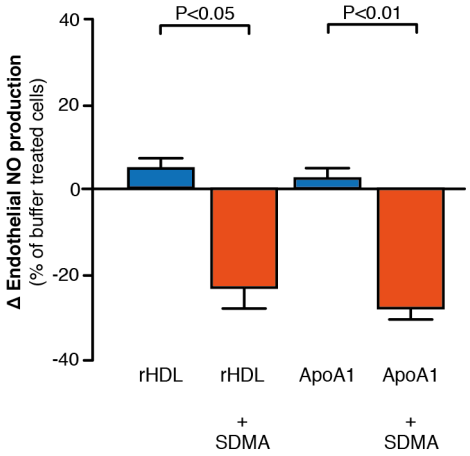

Figure 18 - Effect of rHDL (50 µg/ml) or Apo-A1 (25 µg/ml) supplemented with SDMA (0.5 µmol/g protein) on endothelial NO production (n=4-6 per group).

HDL from healthy subjects is known to stimulate endothelial NO production by Akt-dependent eNOS phosphorylation via activation of endothelial scavenger receptor B-I (SR-BI) or sphingosine-1 phosphate receptors (Acton et al. 1996; Nofer, van der Giet et al. 2004). Here, we found – in contrast to $HDL^{Healthy}$ - that HDL^{CKD} as well as HDL^{SDMA} significantly reduced phosphorylation of Akt (Ser473) leading to a reduced eNOS-activating phosphorylation (Ser1177) and a significantly enhanced eNOS-inhibiting phosphorylation (Thr495).

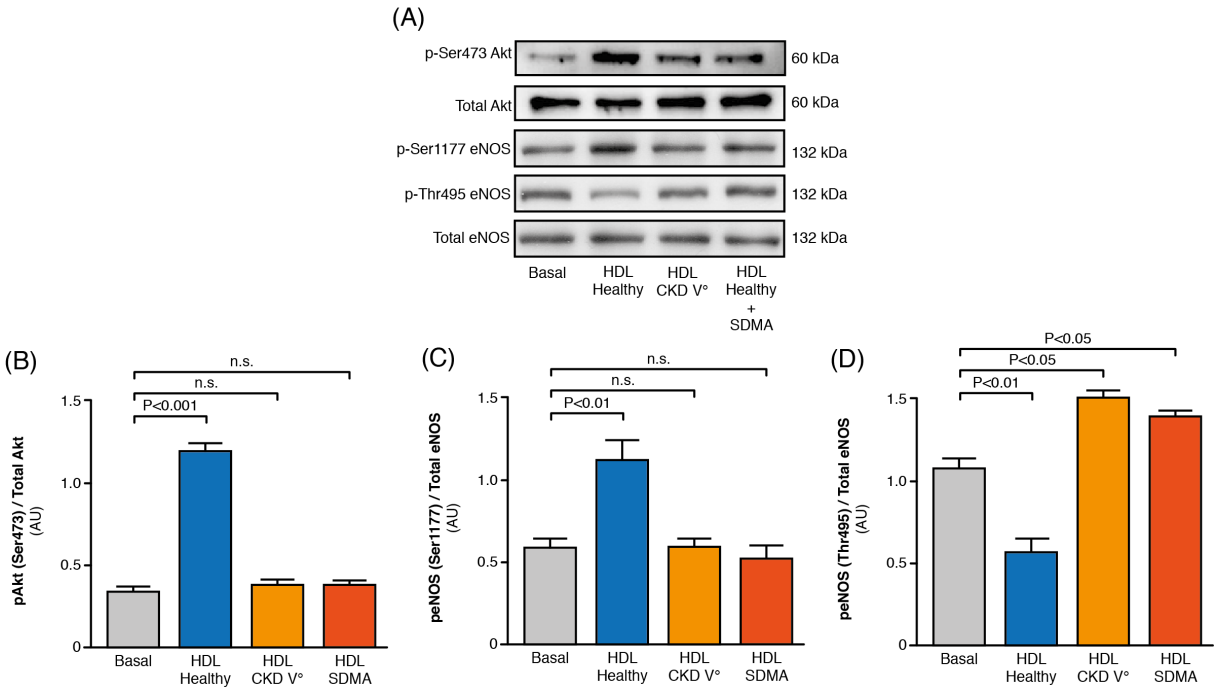

Figure 19 – (A) Phosphorylation of Akt at Ser473, eNOS activating phosphorylation at Ser1177 and eNOS inhibiting phosphorylation at Thr495 determined by western blot analysis in HAEC incubated with HDL (50 µg/ml) for 10 min as indicated (blot as representative of at least 3 independent experiments). (B) Quantification of Akt phosphorylation at Ser473 in HAEC incubated with HDL (50 µg/ml) for 10 min normalized to total Akt expression (n=3-6). (C) Quantification of eNOS-activating phosphorylation at Ser1177 in HAEC incubated with HDL (50 µg/ml) for 10 min normalized to total eNOS expression (n=3-6 per group). (D) Quantification of eNOS-inhibiting phosphorylation at Thr495 in HAEC incubated with HDL (50 µg/ml) for 10 min normalized to total eNOS expression (n=3-6 per group).

Therefore, we hypothesized that such modified HDL changes its affinity to these endothelial receptors mediating the protective endothelial effects of $HDL^{Healthy}$.

Interestingly, binding of $HDL^{Healthy}$ *and* HDL^{SDMA} to endothelial cells did not differ significantly. However, the interaction of HDL^{SDMA} at 37 °C (i.e. cell association) was significantly lower compared to $HDL^{Healthy}$ indicating that SDMA changes the association of HDL with endothelial cells.

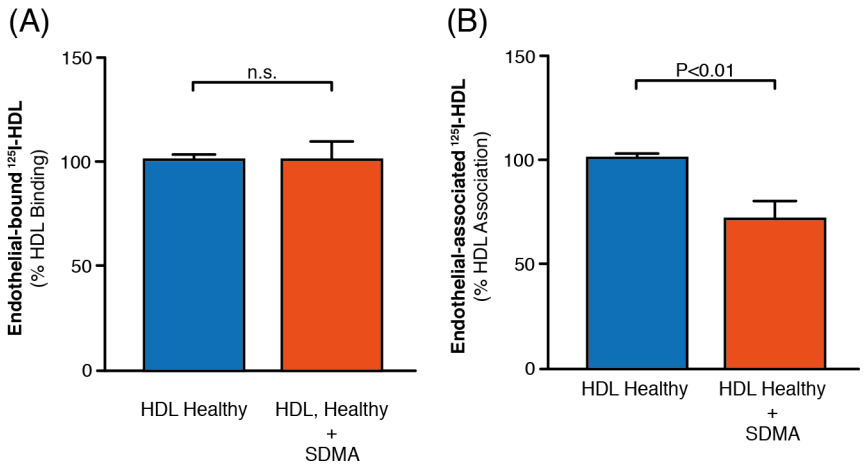

Figure 20 – (A) Effect of SDMA-supplementation on endothelial binding of 125I-HDL (n=6 per group). (B) Effect of SDMA-supplementation on endothelial association of 125I-HDL (n=4 per group).

To further examine the binding affinity of HDL^{SDMA} to the HDL receptor SR-BI, we performed additional binding studies using HEK293 cells transfected with hSRB-I or with an empty vector (control). Here, binding of $HDL^{Healthy}$ and HDL^{SDMA} to HEK cells transfected with hSR-BI did not differ significantly. We repeated the experiment using rHDL supplemented with and without SDMA. Also here, we did not observe a difference in the binding capacity of $HDL^{Healthy}$ and HDL^{SDMA} to SR-BI.

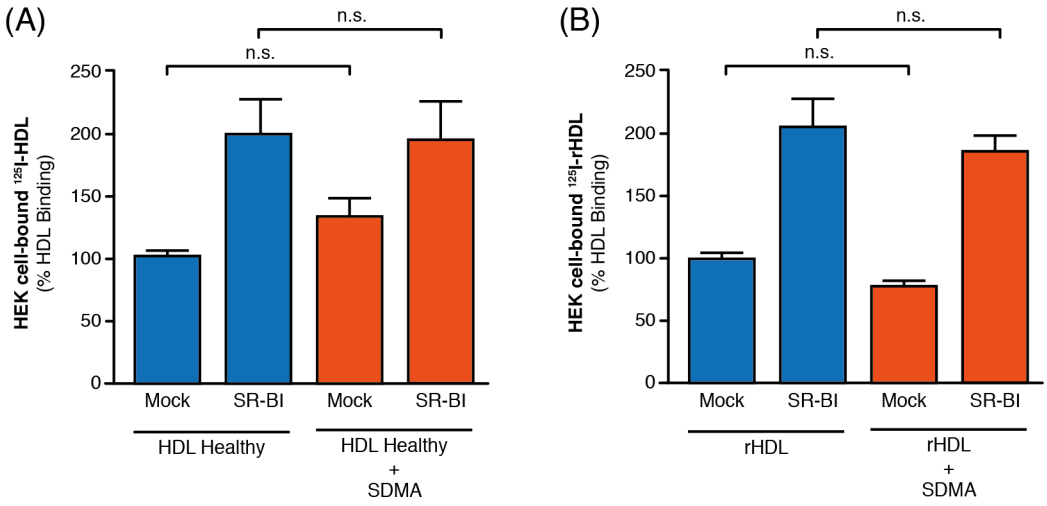

Figure 21 – (A) Binding of ^{125}I-HDL supplemented with SDMA (0.5 µmol/g lipoprotein) to HEK cells untransfected or transfected with human SR-BI (n=7-13 per group). (B) Binding of ^{125}I-rHDL supplemented with SDMA (0.5 µmol/g lipoprotein) to HEK cells untransfected or transfected with human SR-BI (n=6-8 per group).

Collectively, supplementation of ^{125}I-labeled HDL with SDMA reduced the association, but not the specific binding to SR-BI or endothelial cells.

5.4 HDL supplemented with SDMA activates endothelial Toll-like receptor 2

TLR-2 and TLR-4 represent receptors of the innate immune system involved in recognition of pathogen-associated lipoproteins (Takeuchi et al. 2010). Both, TLR-2 and TLR-4 are expressed on HAECs.

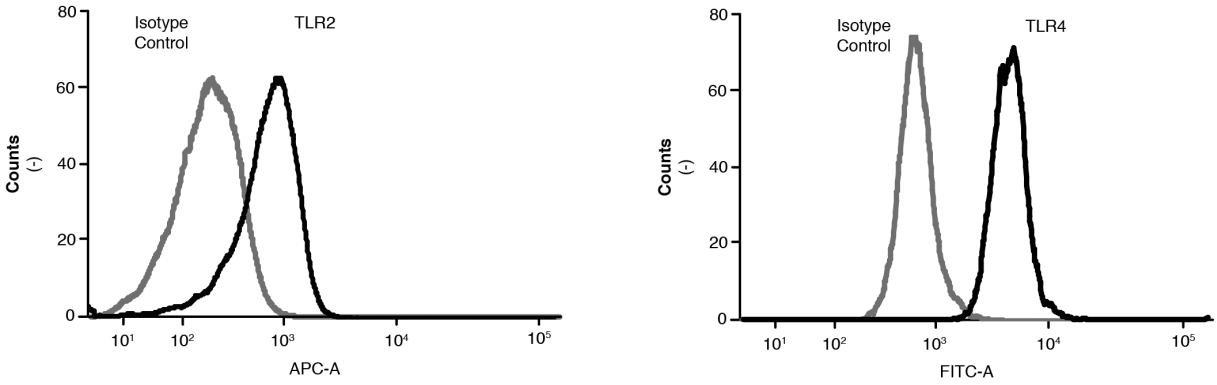

Figure 22 - Representative histogram of TLR-2 and TLR-4 surface expression on HAEC determined by flow-cytometry.

We measured endothelial NO production in HAECs stimulated with $HDL^{Healthy}$, HDL^{CKD} or HDL^{SDMA} in the presence or absence of specific neutralizing antibodies against TLR-2 and TLR-4. We observed that blocking of TLR-2 but not TLR-4 restored the effects of HDL^{CKD} and HDL^{SDMA} on endothelial NO production. Importantly, the neutralizing antibodies did not affect NO production in the presence of $HDL^{Healthy}$.

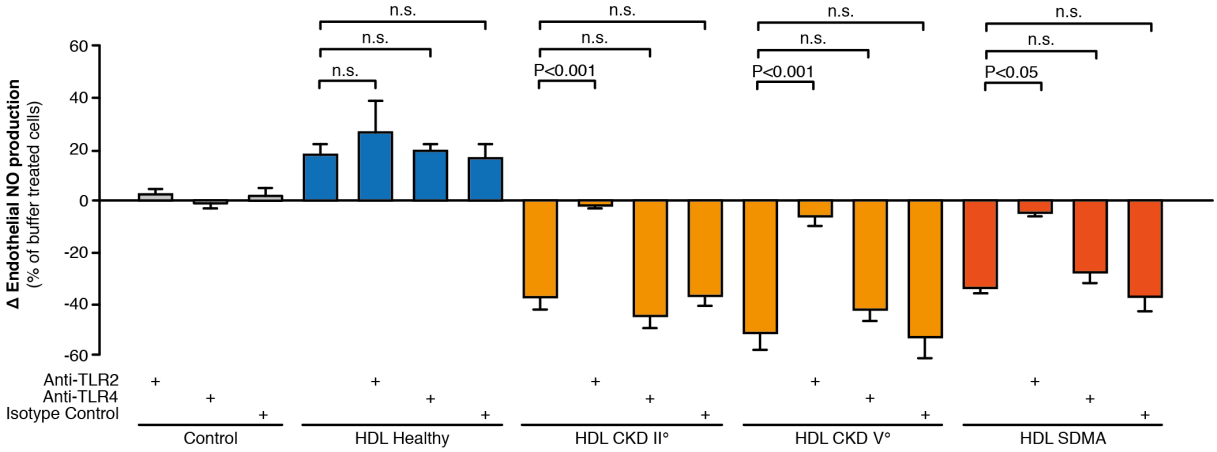

Figure 23 - Effect of TLR-2 and TLR-4 inhibition using a blocking antibody (10 μg/ml, each) or an isotype control antibody (10 μg/ml) on endothelial NO production after incubation with HDLHealthy, HDLCKD and HDLSDMA (0.5 μmol/g SDMA, 50 μg/ml HDL, n=3-5 per group).

Consistently, injection of *HDL*^{CKD} into *Tlr2*$^{-/-}$ mice, but not in *Tlr4*$^{-/-}$ mice, failed to increase ABP.

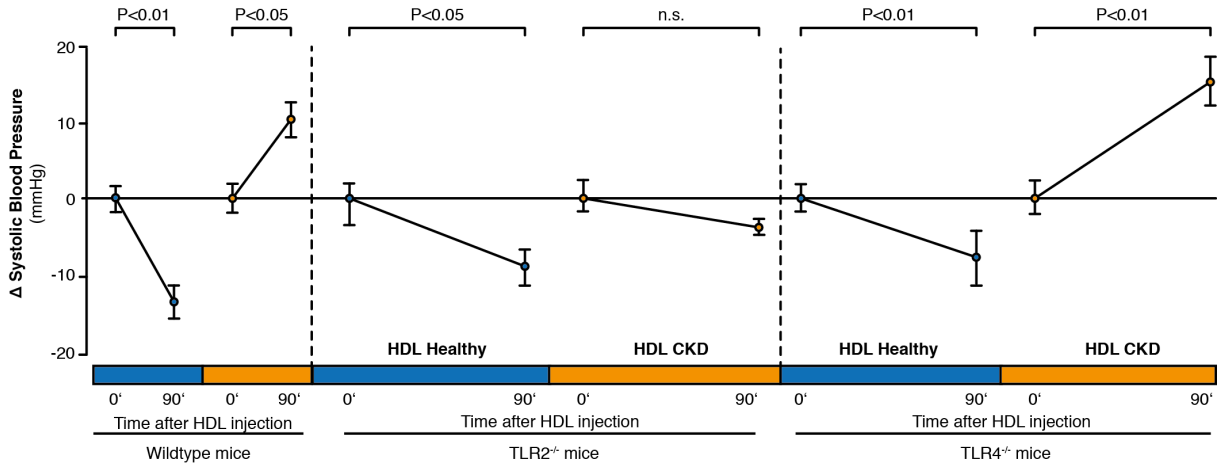

Figure 24 - Δ ABP in *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice 90 min after i.v. injection of *HDL*^{Healthy} and *HDL*^{CKD} (15 mg/kg HDL, n=3-6 per group).

To examine whether TLR-2 on circulating mononuclear cells contributes to the effect of *HDL*^{CKD} on ABP, we analyzed wildtype (WT) and *Tlr2*$^{-/-}$ bone-marrow chimeric mice, which were sublethally irradiated and transplanted with bone-marrow from WT or *Tlr2*$^{-/-}$ mice. We observed an increased ABP in *HDL*^{CKD}-treated mice with intact TLR-2 expression on vascular cells, but lack on bone-marrow derived circulating mononuclear cells. These data suggest, that TLR-2 on the endothelium but not on circulating mononuclear cells mediates the adverse effects of *HDL*^{CKD} on ABP.

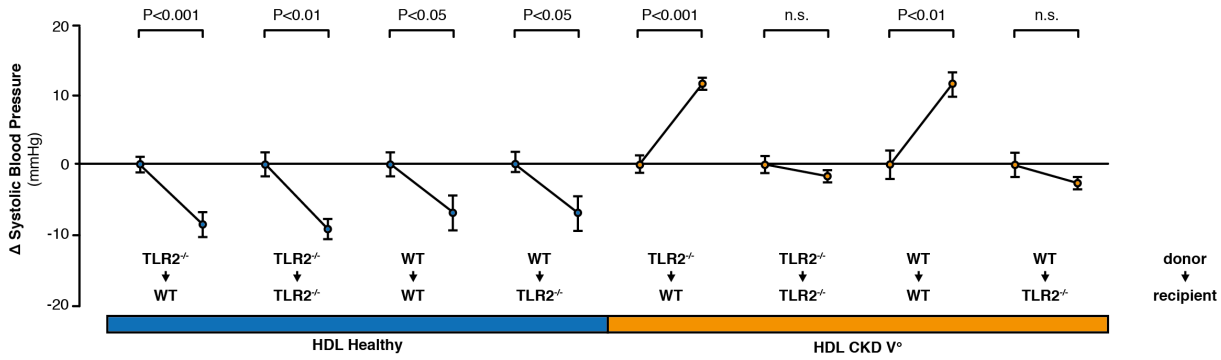

Figure 25 - Δ ABP 90 min after i.v. injection of *HDL*^{Healthy} and *HDL*^{CKD} (15 mg/kg HDL) in wildtype, *Tlr2*$^{-/-}$ mice 6 weeks after bone-marrow transplantation from wildtype and *Tlr2*$^{-/-}$ mice as indicated (n=3 per group).

Of note, the response of ABP did not differ between WT and *Tlr2*$^{-/-}$ mice after injection of the eNOS inhibitor L-NAME.

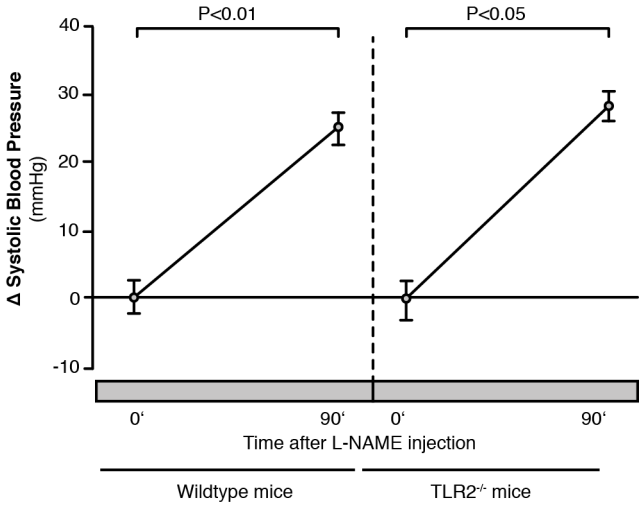

Figure 26 - Δ ABP in wildtype and *Tlr2*$^{-/-}$ mice 30 min after i.v. injection of L-NAME (17 mg/kg, n=3-6 per group).

To assess the interaction between *HDL*^{SDMA} and TLR-2, we transfected endothelial cells with a plasmid encoding for human TLR-2 without its intracellular TIR-domain (TLR2-ΔTIR-HA) and incubated them with fluorescent-labeled HDL. As control, we used a plasmid encoding human TLR-5 (TLR2-ΔTIR-HA), which is also expressed on the cell surface (Ramos et al. 2004). Overexpression of TLR-2 increased the interaction of endothelial cells with *HDL*^{SDMA}, whereas overexpression of TLR-5 did not change the interaction with HDL and endothelial cells.

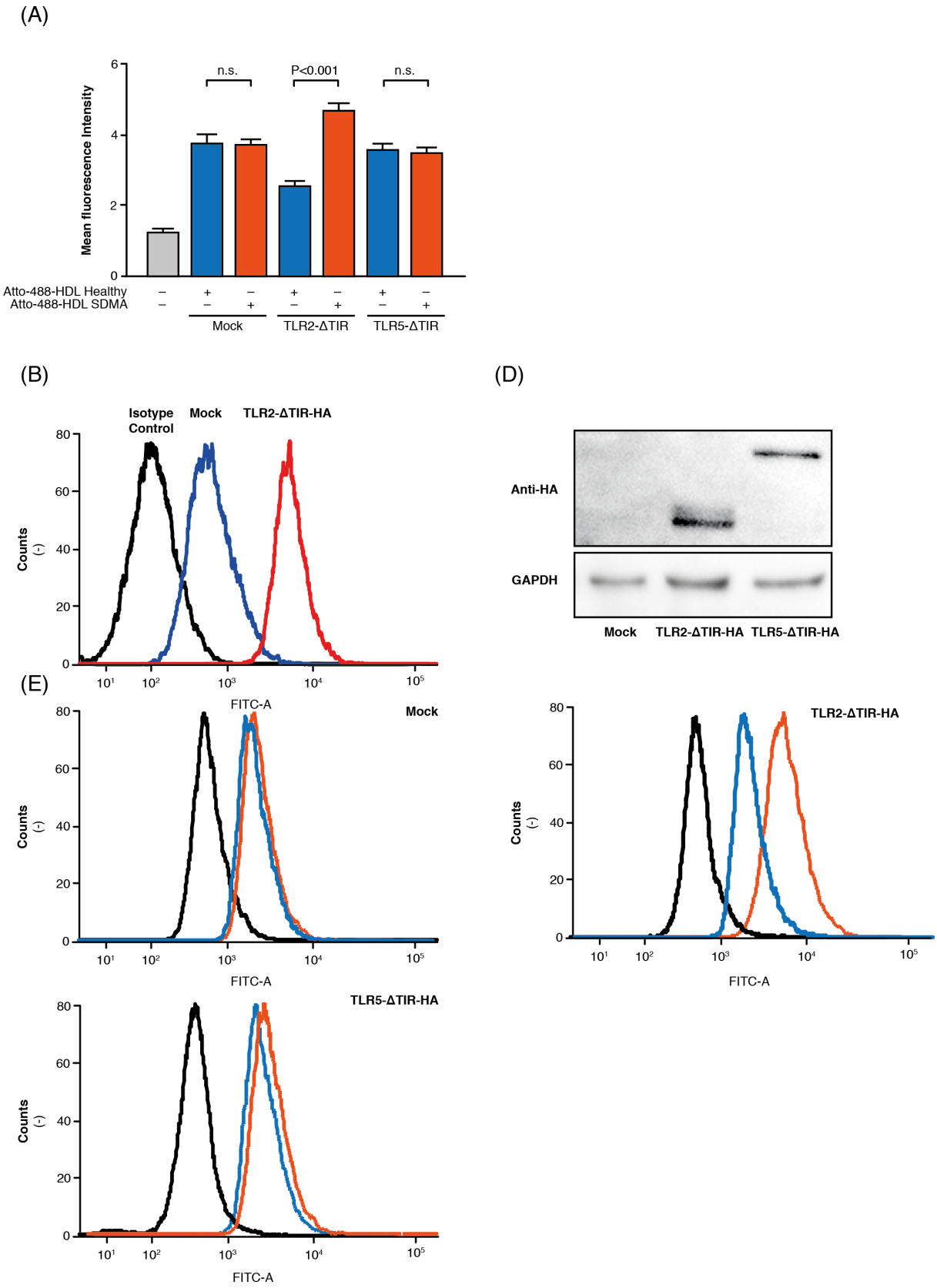

Figure 27 – (A) Interaction of Atto-488-labeled HDL (20 μg/ml, 30 min) with endothelial cells transfected with a plasmid containing hTLR2-ΔTIR-HA or hTLR5-ΔTIR-HA. Mean fluorescence was recorded using flow-cytometry (n=6 per group). (B) Representative histogram of TLR-2 surface expression on HAEC 48 hrs after transfection with an empty vector (blue) or a TLR2-ΔTIR-HA encoding vector (red) determined by flow-cytometry. (C) Representative western blot using an Anti-HA antibody 48 hrs after transfection of HAEC with an empty vector (mock), a vector encoding TLR2-ΔTIR-HA or a vector encoding TLR5-ΔTIR-HA. (D) Representative histograms of HAEC transfected with an empty vector (mock), a vector encoding

TLR2-ΔTIR-HA or a vector encoding TLR5-ΔTIR-HA treated for 30 min with 20 μg/ml Atto-488 labeled *HDL*^{Healthy} (blue) or *HDL*^{SDMA} (red) determined by flow-cytometry.

5.5 Modified HDL does not activate NF-κB dependent TLR-2 signaling

Next, we asked whether activation of TLR-2 in the absence of *HDL*^{CKD} or *HDL*^{SDMA} is sufficient to inhibit endothelial NO production. Pam3CSK4 and FSL-1 are synthetic lipopeptides, known to bind to TLR-2 (Fujita et al. 2003; Jin et al. 2007). Treatment of HAECs with Pam3CSK4 or FSL-1 inhibited endothelial NO production. This effect was completely abolished in the presence of anti-TLR-2 neutralizing antibodies. In contrast, the TLR-4 agonist LPS increased endothelial NO production in a TLR-4 dependent manner.

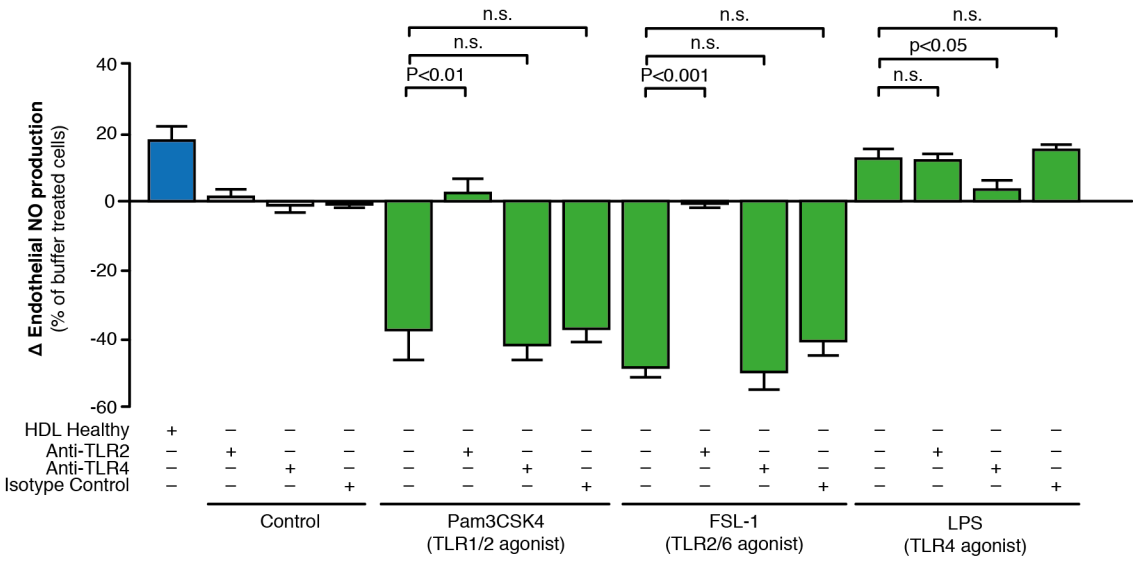

Figure 28 - Endothelial NO production after stimulation (1 hr) with the TLR-1/2 agonist Pam3CSK4 (1 μg/ml), TLR2/6 agonist FSL-1 (100 ng/ml) and the TLR-4 agonist LPS (1 μg/ml) with or without preincubation with blocking antibodies against TLR-2 or TLR-4 (10 μg/ml), (n=3-5 per group).

In vivo, a single-dose of Pam3CSK4 was sufficient to significantly increase ABP in WT, but not in *Tlr2*$^{-/-}$ mice.

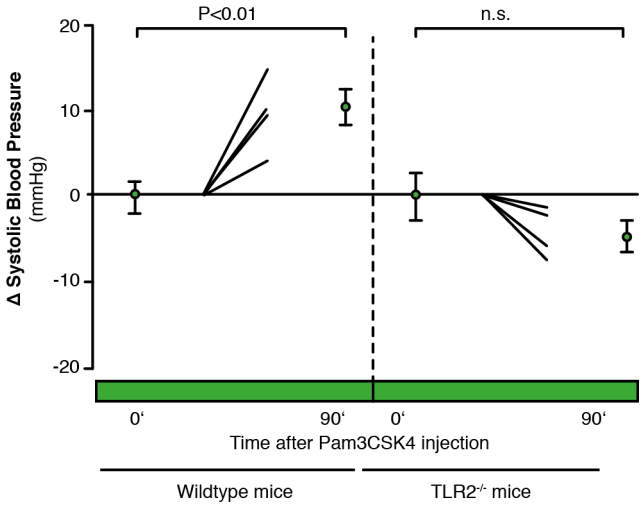

Figure 29 - Δ Systolic ABP in wildtype and *Tlr2*$^{-/-}$ mice 90 min after i.v. injection of Pam3CSK4 (5 mg/kg, n=4 per group).

Recognition of bacterial lipoproteins usually requires formation of heterodimer complexes of TLR-2 with TLR-1 or TLR-6, respectively (Alexopoulou et al. 2002; Fujita, Into et al. 2003; Sandor et al. 2003). Pam3CSK4 is recognized by the TLR-1/TLR-2 and FSL-1 by the TLR-2/TLR-6 heterodimer resulting in NF-κB pathway activation and proinflammatory cytokine release (Beutler 2004). We used human embryonic kidney (HEK)-Blue TLR-2 reporter cells to address if modified HDL activates the NF-κB pathway. Surprisingly, in contrast to Pam3CSK4 and FSL-1, both *HDL*^{CKD} and *HDL*^{SDMA} failed to activate NF-κB pathway in the reporter cells under basal conditions.

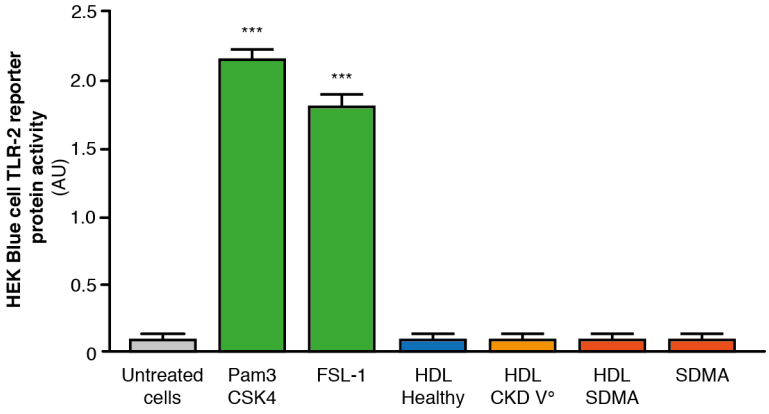

Figure 30 - TLR-2 activity in HEK cells cotransfected with TLR-2 and SEAP under control of NF-κB and AP-1 after stimulation with Pam3CSK4 (1 μg/ml), FSL-1 (100 ng/ml), HDL (50 μg/ml) or SDMA (4 μmol/L) for 18 hrs (n=4 per group, * *P*<0.001 compared to untreated cells).**

Pam3CSK4 induced release of cytokines in bone-marrow derived macrophages from wildtype, but not from *Tlr2*$^{-/-}$ mice, and in human peripheral blood mononuclear cells (PBMC). In contrast, production of these cytokines was not stimulated by *HDL*^{CKD} and *HDL*^{SDMA}. Notably, neither Pam3CSK4 nor HDL did affect cytokine release in HAEC as determined by ELISA or intracellular cytokine staining.

	Untreated	Pam3CSK4	HDL^{Healthy}	HDL$^{CKD-V°}$	HDL^{SDMA}	SDMA
TNF (pg/ml)						
BM-Mθ (WT)	< 4	1030 ± 24	< 4	< 4	< 4	< 4
BM-Mθ (*Tlr2*$^{-/-}$)	< 4	< 4	< 4	< 4	< 4	< 4
PBMC	< 4	426 ± 4	< 4	< 4	< 4	< 4
HAEC	< 4	< 4	< 4	< 4	< 4	< 4
IL-1β (pg/ml)						
BM-Mθ (WT)	< 4	107 ± 12	< 4	< 4	< 4	< 4
BM-Mθ (*Tlr2*$^{-/-}$)	< 4	< 4	< 4	< 4	< 4	< 4
PBMC	< 4	49 ± 3	< 4	< 4	< 4	< 4
HAEC	< 4	< 4	< 4	< 4	< 4	< 4
IL-10 (pg/ml)						
BM-Mθ (WT)	< 2	289 ± 19	< 2	< 2	< 2	< 2
BM-Mθ (*Tlr2*$^{-/-}$)	< 2	< 2	< 2	< 2	< 2	< 2
PBMC	6 ± 1	229 ± 49	6 ± 1	7 ± 1	6 ± 1	8 ± 1
HAEC	< 2	< 2	< 2	< 2	< 2	< 2
IL-12 p40 (pg/ml)						
BM-Mθ (WT)	< 62	1194 ± 47	< 62	< 62	< 62	< 62
BM-Mθ (*Tlr2*$^{-/-}$)	< 62	< 62	< 62	< 62	< 62	< 62
PBMC	< 62	1400 ± 130	< 62	< 62	< 62	< 62
HAEC	< 62	< 62	< 62	< 62	< 62	< 62
IL-6 (pg/ml)						
BM-Mθ (WT)	< 2	137 ± 16	< 2	< 2	< 2	< 2
BM-Mθ (*Tlr2*$^{-/-}$)	< 2	< 2	< 2	< 2	< 2	< 2
PBMC	< 2	55 ± 11	7 ± 1	7 ± 1	8 ± 5	7 ± 1
HAEC	3 ± 1	3 ± 1	4 ± 1	5 ± 1	< 2	3 ± 1

Table 5 - Production of IL-1β, IL-6, IL-10, TNF, IL-12 (p40) in bone-marrow derived macrophages from wildtype (BM-MΦ WT) and *Tlr2*$^{-/-}$ mice (BM-MΦ *Tlr2*$^{-/-}$), human PBMC and HAEC stimulated with Pam3CSK4, *HDL*^{Healthy}, *HDL*$^{CKD-V°}$, *HDL*^{SDMA} and SDMA for 24 hours.

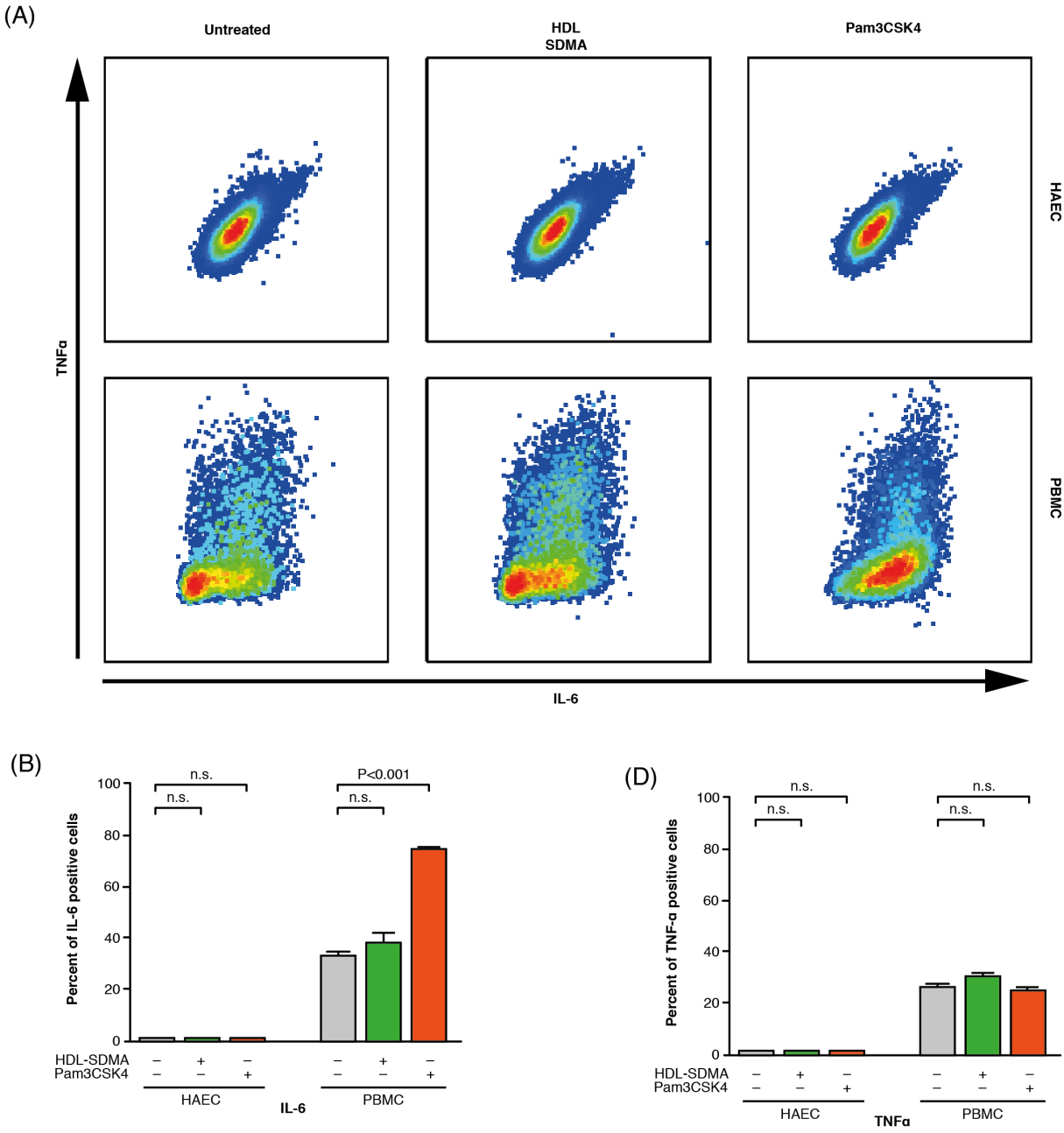

Figure 31 – (A) Intracellular cytokine staining for TNFα and IL-6 in HAEC and human PBMC incubated with *HDL*^{SDMA} (50 µg/ml) or Pam3CSK4 (1 µg/ml) for 6 hours determined by flow-cytometry (representative for n=3 independent experiments). (B) Mean fluorescence intensity after intracellular cytokine staining for IL-6 in HAEC and human PBMC (n=3). (C) Mean fluorescence intensity after intracellular cytokine staining for TNF-α in HAEC and human PBMC (n=3).

Furthermore, we used neutralizing antibodies against TLR-1, TLR-2, and TLR-6 to block NF-κB activation in the HEK-Blue TLR-2 reporter cells stimulated with Pam3CSK4 and FSL-1. As expected, blocking of either TLR-1 or TLR-6 and TLR-2 almost completely abolished Pam3CSK4 or FSL-1 induced NF-κB activation.

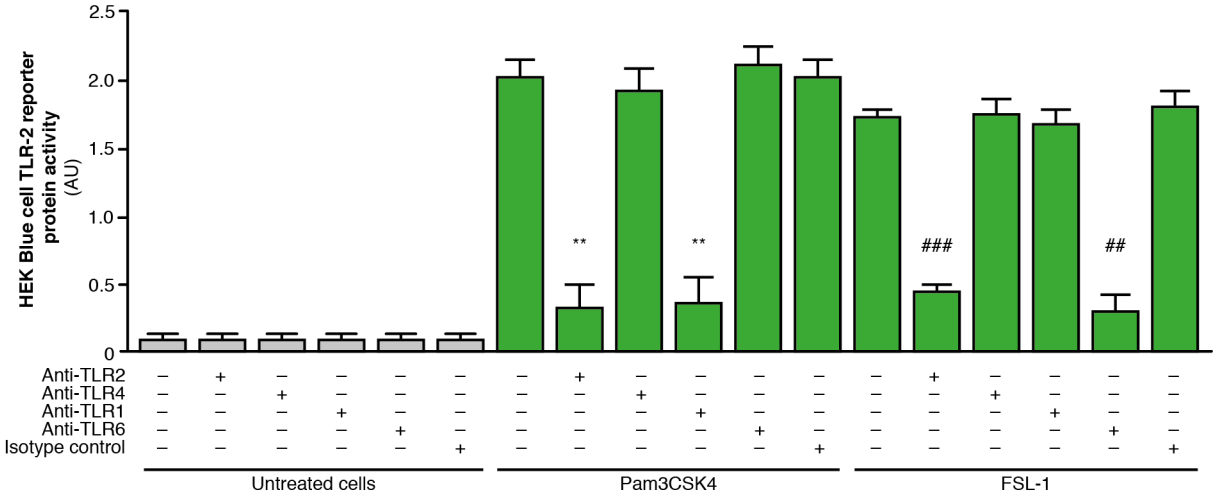

Figure 32 - TLR-2 activity in HEK cells cotransfected with TLR-2 and SEAP under control of NF-κB and AP-1 after preincubation with blocking antibodies targeting TLR-2, TLR-4, TLR-1, TLR-6 or isotype control (10 μg/ml) for 1 hr and subsequent stimulation with Pam3CSK4 (1 μg/ml) or FSL-1 (100 ng/ml) for 1 hr (n=4 per group, ** *P*<0.01 compared to Pam3CSK4 treated cells, ### *P*<0.001 and ## *P*<0.01 compared to FSL-1 treated cells).

5.6 TLR-1- or TLR-6-co-receptor activation is not required to induce TLR-2 mediated endothelial dysfunction

We determined the presence of TLR-1- and TLR-6-co-receptors on HAEC by flow-cytometry. Of note, in contrast to human PBMC, HAEC only expressed TLR-2 on their surface, while TLR-1 and TLR-6 were not detectable.

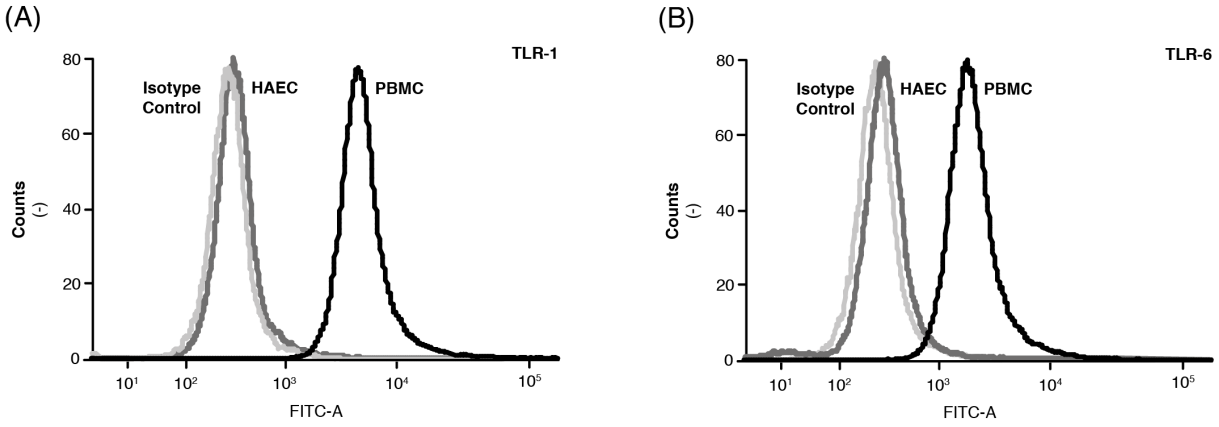

Figure 33 - (A) Representative histogram of TLR-1 surface expression on HAEC and human PBMC determined by flow-cytometry. (B) Representative histogram of TLR-6 surface expression on HAEC and human PBMC determined by flow-cytometry.

Next, we addressed whether TLR-1 or TLR-6 blockade can restore NO production in HAEC treated with Pam3CSK4 or HDL^{CKD}. In contrast to TLR-2 blockade, addition of anti-TLR-1

and/or anti-TLR-6 neutralizing antibodies had no effect on endothelial NO production in the presence of Pam3CSK4, HDL^{CKD} or $HDL^{Healthy}$.

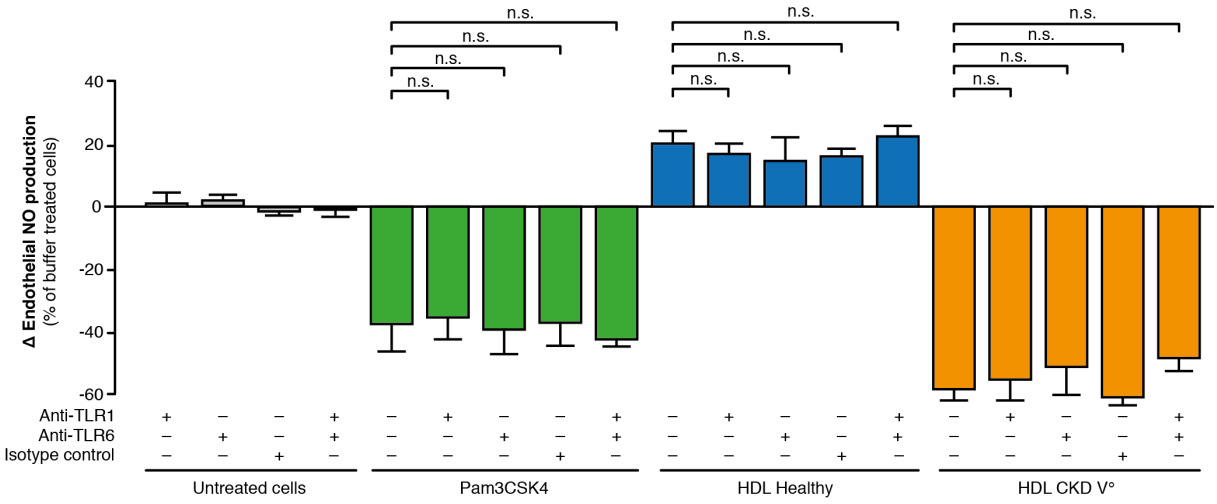

Figure 34 - Endothelial NO production after preincubation with TLR-1 and/or TLR-6 neutralizing antibody or an isotype control antibody (10 µg/ml, 1 hr) and subsequent stimulation with Pam3CSK4 (1 µg/ml) or HDL (50 µg/ml, 1 hr, n=3-6 per group).

Collectively, our data suggest that modified HDL activates TLR-2 inducing a TLR-1- and TLR-6-co-receptor independent pathway in endothelial cells. Consistently, absence of TLR-1 (*Tlr1*$^{-/-}$ mice) and TLR-6 (*Tlr6*$^{-/-}$ mice) *in vivo* did not prevent the effect of HDL^{CKD} on ABP.

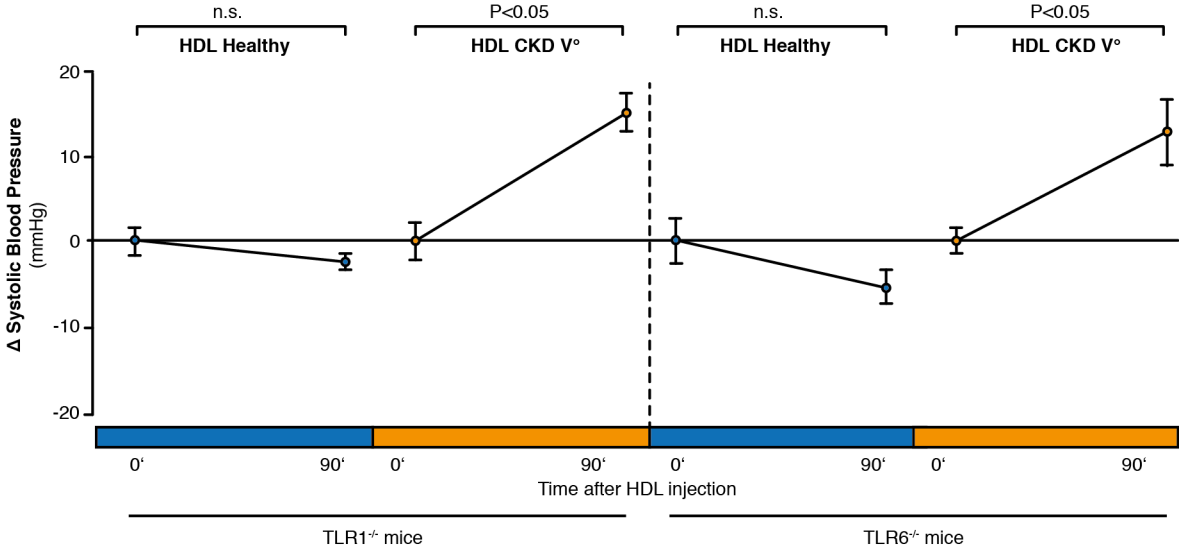

Figure 35 - Δ Systolic ABP in *Tlr1*$^{-/-}$ and *Tlr6*$^{-/-}$ mice 90 min after i.v. injection of $HDL^{Healthy}$ and HDL^{CKD} (15 mg/kg, n=4 per group).

Furthermore, we analyzed endothelial superoxide production in response to Pam3CSK4 and FSL-1 in the presence or absence of neutralizing antibodies against TLR-2, TLR-1 or TLR-6.

We observed elevated superoxide levels in HAEC treated with Pam3CSK4 and FSL-1. Blockade of TLR-2, but not TLR-1 and/or TLR-6, prevented this effect.

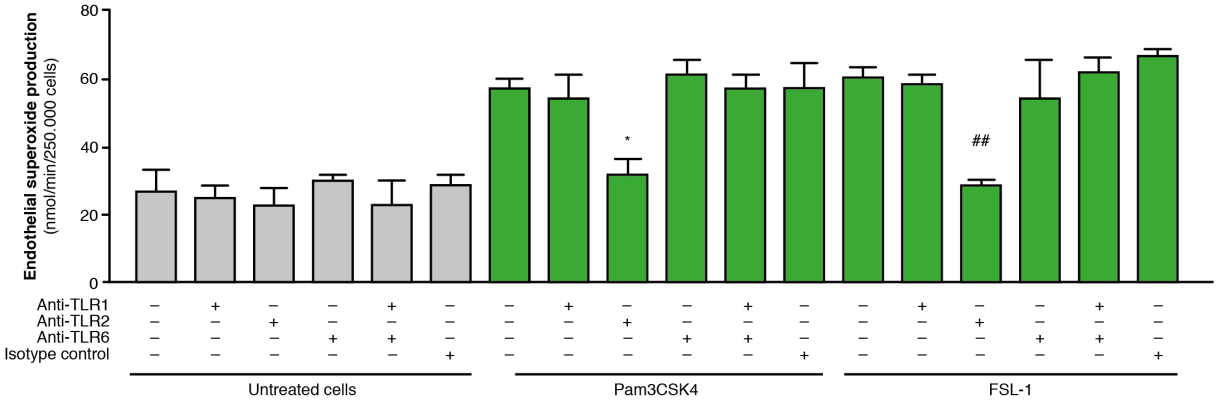

Figure 36 - Endothelial superoxide production after preincubation with a TLR-1, TLR-2 or TLR-6 neutralizing antibody or an isotype control (10 μg/ml, 1 hr) and stimulation with Pam3CSK4 or FSL-1 (1 μg/ml or 100 ng/ml, 1 hr; * *P*<0.05 compared to Pam3CSK4 treated cells, ## P<0.01 compared FSL-1 treated cells, n=3-6 per group).

5.7 Activation of endothelial TLR-2 inhibits eNOS activating pathways and stimulates NADPH oxidase

Next, we assessed the molecular mechanisms on how TLR-2 activation affects endothelial superoxide and nitric oxide production. At first, we analyzed the effect of the TLR-2 agonist Pam3CSK4 on endothelial Akt and eNOS phosphorylation. Comparable to HDL^{CKD} and HDL^{SDMA}, Pam3CSK4 reduced Akt phosphorylation at Ser473 and, thereby, induced eNOS-inhibiting phosphorylation at Thr495.

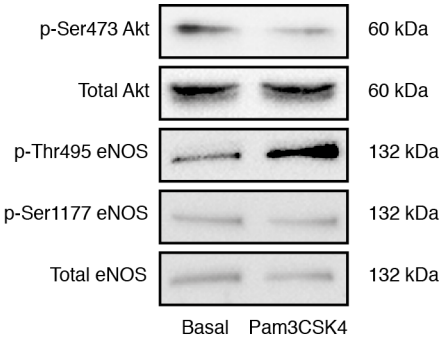

Figure 37 - Phosphorylation of Akt at Ser473, eNOS activating phosphorylation at Ser1177 and eNOS inhibiting phosphorylation at Thr495 determined by western blot analysis in HAEC incubated with Pam3CSK4 (1 μg/ml) for 10 min (blot as representative of 3 independent experiments).

In addition, it has been shown that TLR-2 activation induces NADPH oxidase to produce ROS in monocytes and macrophages (Beaulieu et al. 2011), however, its role in endothelial

cells remained unclear. By measuring endothelial superoxide production after preincubation with a specific chemical inhibitor of NADPH oxidase (VAS-2870), we demonstrated a pivotal role for NADPH oxidase in TLR-2 induced endothelial superoxide production. Using L-NAME, an eNOS inhibitor, we ruled out eNOS uncoupling as an immediate source of TLR-2 mediated superoxide production.

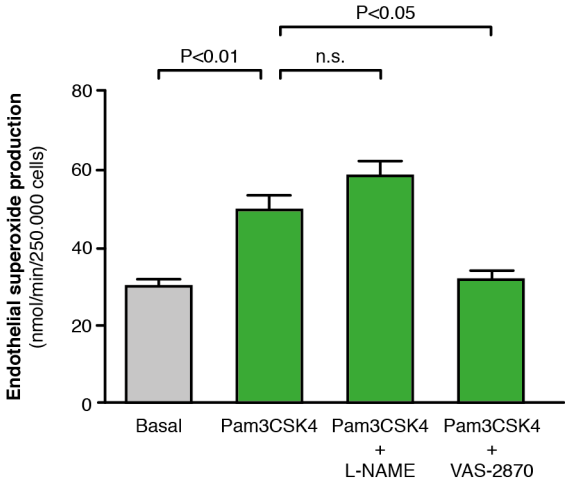

Figure 38 - Endothelial superoxide production after preincubation with L-NAME (0.3 mM, 1 hr), a specific eNOS inhibitor, or VAS-2870 (10 μM, 1 hr), a specific NAPDH-oxidase inhibitor and subsequent stimulation with Pam3CSK4 (1 μg/ml; 1 hr, n=3-6 per group).

Notably, inhibition of NADPH oxidase almost completely prevented the effect of *HDL*^{CKD}, *HDL*^{SDMA} and Pam3CSK4 on endothelial nitric oxide production.

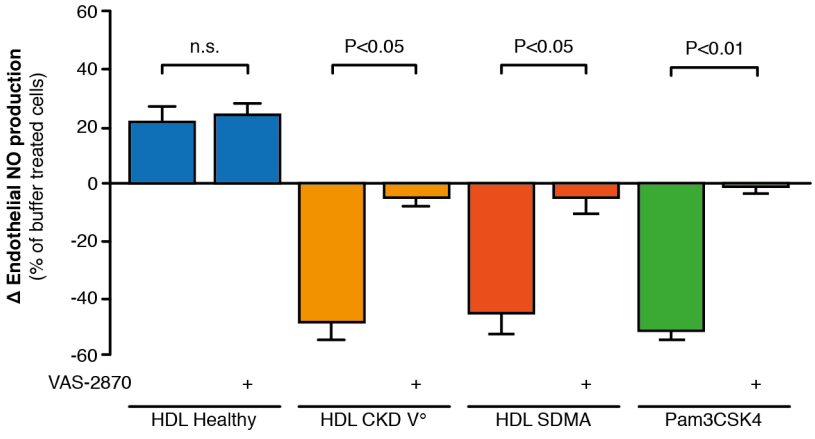

Figure 39 - Endothelial nitric oxide production after stimulation with Pam3CSK4 (1 μg/ml) or HDL (50 μg/ml) with or without preincubation with VAS-2870 (10 μM, 1 hr, n=3 per group).

TLR-2 activation is known to induce phosphorylation of c-Jun N-terminal kinase (JNK) and we have recently shown that activation of JNK in endothelial cells induces endothelial superoxide production by increasing NADPH oxidase activity (Cabanski et al. 2008; Osto et

al. 2008; Shi et al. 2011). Here, we observed that Pam3CSK4 induces JNK phosphorylation in HAEC.

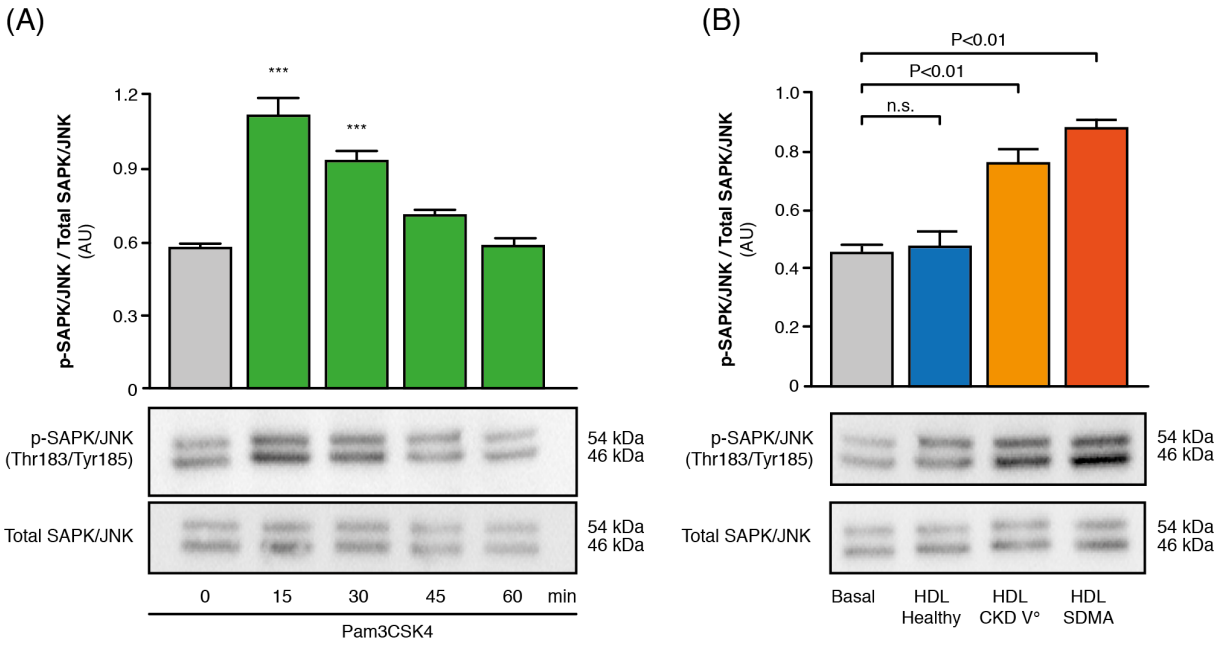

Figure 40 – (A) Time-dependent effect of TLR-2 activation with Pam3CSK4 (1 µg/ml) on endothelial SAPK/JNK phosphorylation (* *P*<0.001 compared to basal, blot as representative of 4 independent experiments). (B) Effect of $HDL^{Healthy}$, HDL^{CKD} and HDL^{SDMA} (50 µg/ml, 15 min) on endothelial SAPK/JNK phosphorylation (n=3 per group).**

Accordingly, SP600125, a JNK inhibitor, abolished the effect of HDL^{CKD}, HDL^{SDMA} and Pam3CSK4 on endothelial superoxide and nitric oxide production.

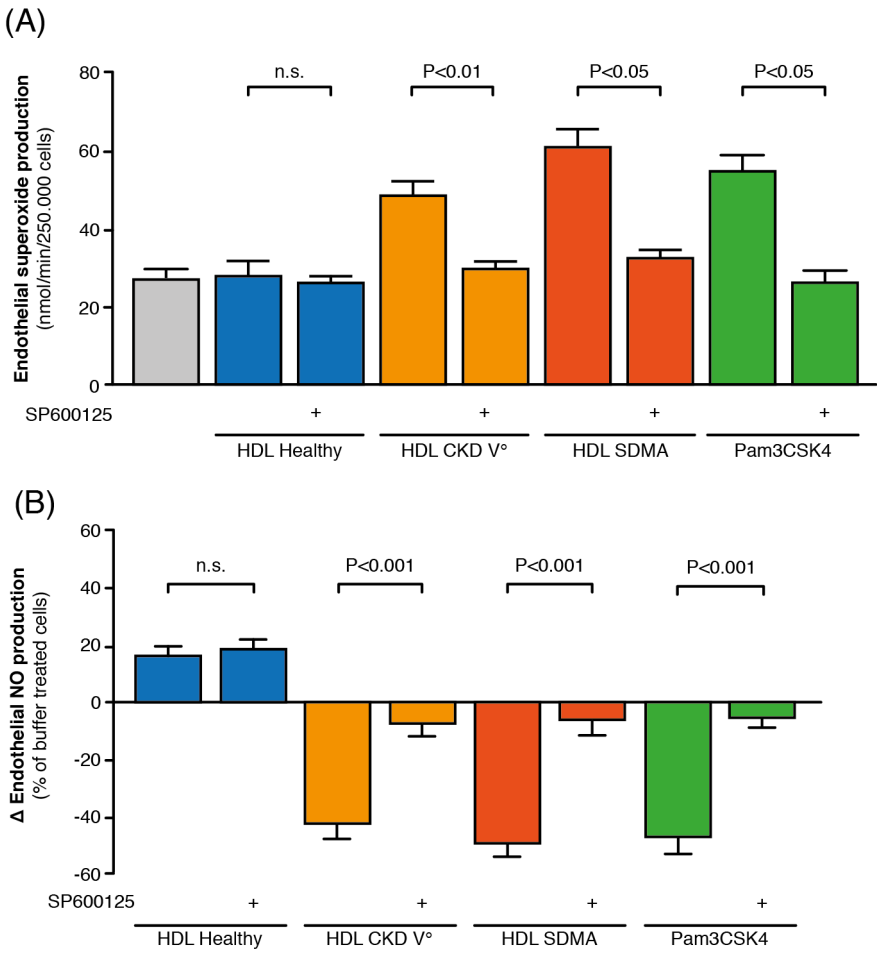

Figure 41 – (A) Endothelial superoxide production after incubation with Pam3CSK4 (1 μg/ml) or HDL (50 μg/ml) for 1 hr with or without preincubation with SP600125, a SAPK/JNK inhibitor (1 μM, n=3-4 per group). (B) Endothelial nitric oxide production after stimulation with Pam3CSK4 (1 μg/ml) or HDL (50 μg/ml) with or without preincubation with SP600125 (1 μM, 1 hr, n=3 per group).

5.8 *HDL^{CKD}* and *HDL^{SDMA}* impair endothelial repair and promote inflammatory activation

We and others have previously demonstrated that reduced NO bioavailability along with enhanced superoxide production may also impair other endothelial functions, such as endothelial repair after injury and its anti-inflammatory capacity (Kubes et al. 1991; Sorrentino, Bahlmann et al. 2007). To elucidate whether modified HDL also affects these endothelial properties by inhibiting endothelial NO production, we finally assessed the effect of modified HDL on endothelial repair and inflammation.

In an endothelial wound healing assay, *HDL^{Healthy}* strongly stimulated endothelial cell migration, whereas *HDL^{CKD}* inhibited endothelial migration.

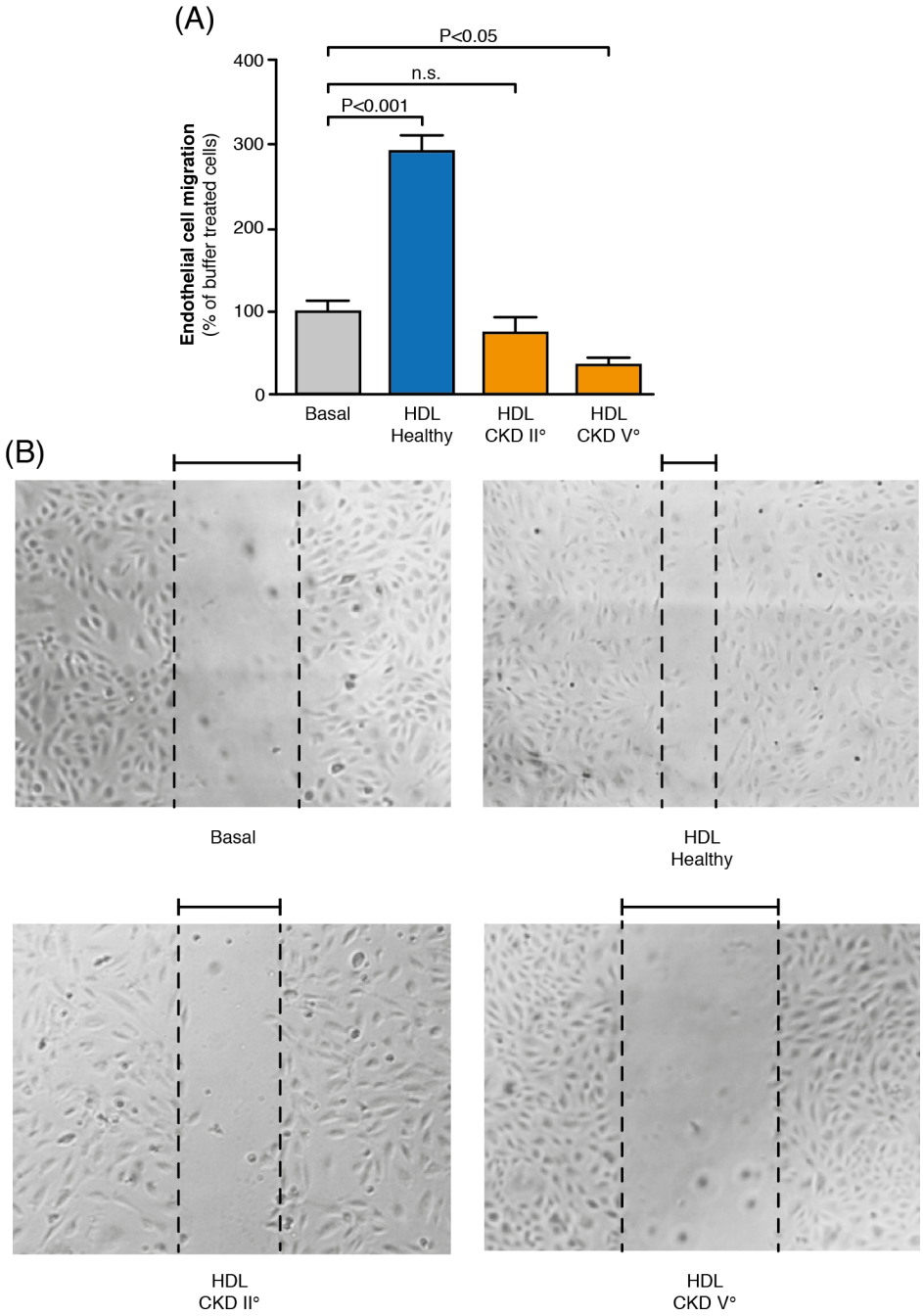

Figure 42 – (A) Endothelial cell migration after incubation with HDL (50 μg/ml, 24 hr) in a scratch assay (n=4-8 per group). (B) Representative micrographs of endothelial cell migration in a scratch assay after incubation with HDL.

In line with these *in vitro* observations, HDL^{CKD} and HDL^{SDMA} inhibited endothelial repair *in vivo* in a carotid artery injury model. This was in marked contrast to $HDL^{Healthy}$, which promoted endothelial repair.

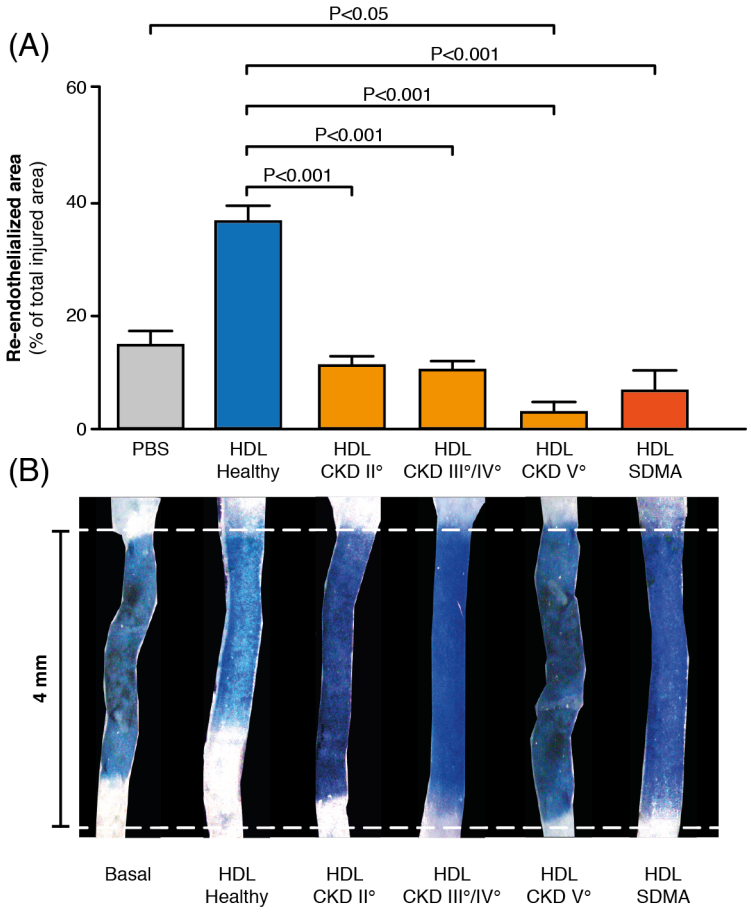

Figure 43 – (A) Reendothelialized area at day 3 after carotid injury and injection of *HDL^{Healthy}*, *HDL^{CKD}* or *HDL^{SDMA}* (15 mg/kg) in nude mice. PBS treated animals serve as control (n=7-13 per group). (B) Representative photographs of carotid arteries after Evans Blue staining.

In *Tlr2$^{-/-}$* mice, we observed that *HDL^{CKD}* regained its capacity to promote endothelial repair.

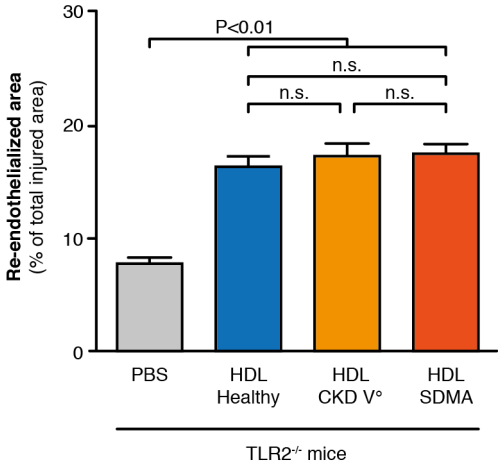

Figure 44 - Reendothelialized area at day 3 after carotid injury in *Tlr2-/-* mice with injection of *HDL^{Healthy}*, *HDL^{CKD}* or *HDL^{SDMA}* (15 mg/kg). PBS treated animals serve as control (n=6 per group).

These findings underscore the important role of TLR-2 to mediate adverse endothelial effects of *HDL^{CKD}* and *HDL^{SDMA}*.

Furthermore, we observed that *HDL*^{CKD} increased endothelial pro-inflammatory activation as determined by a higher amount of adhering mononuclear cells to a TNFα-stimulated endothelial monolayer, which was in contrast to *HDL*^{Healthy}. Accordingly, *HDL*^{CKD} and *HDL*^{SDMA} increased endothelial VCAM-1 expression, while *HDL*^{Healthy} significantly reduced VCAM-1 expression on endothelial cells.

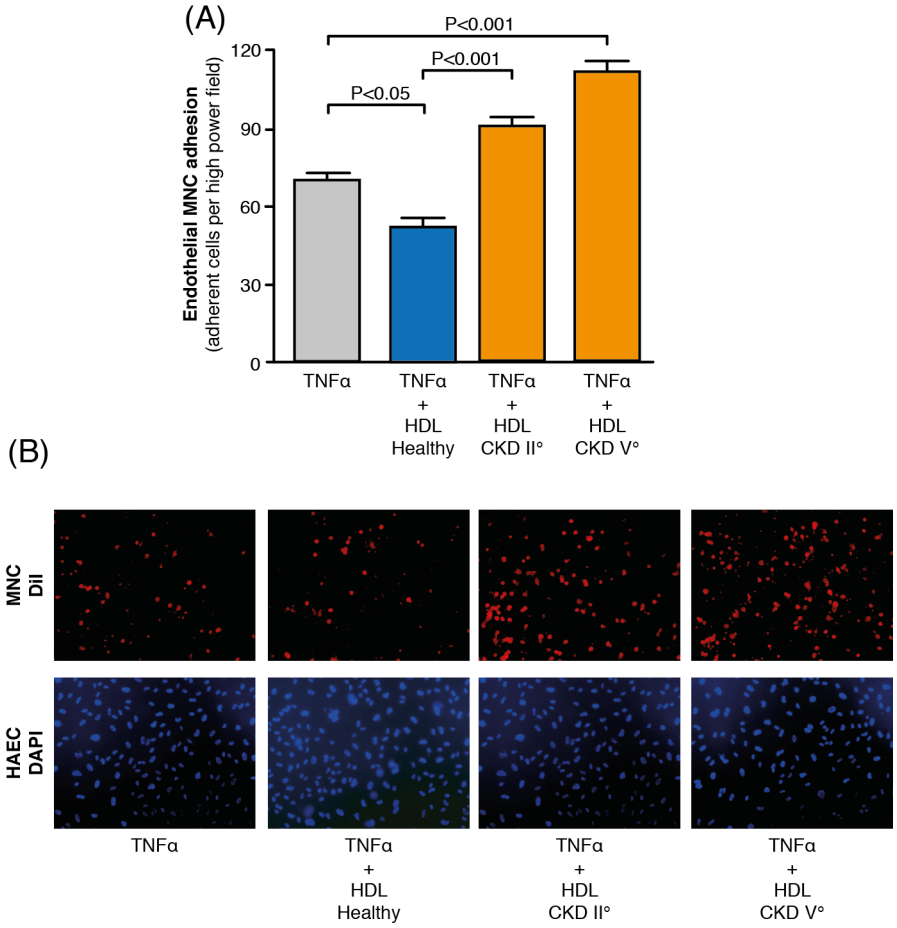

Figure 45 – (A) Adhesion of mononuclear cells (MNC) to TNF-α (5 ng/ml) treated endothelial cells after incubation with *HDL*^{Healthy} or *HDL*^{CKD} (50 µg/ml). MNC are stained with DiI and HAEC with DAPI (n=6 per group). (B) Representative micrographs of high-power fields of 6 independent experiments obtained by fluorescence microscopy.

Next, we analyzed the effect of NO on TNFα induced VCAM-1 expression in the presence of HDL. Inhibition of eNOS through L-NAME reduced the anti-inflammatory effects of *HDL*^{Healthy} by enhancing endothelial VCAM-1 expression, whereas supplementation of NO using a solid NO-donor, diethylenetriamine/nitric oxide (DetaNO), restored the anti-inflammatory function of the endothelium by reducing endothelial VCAM-1 expression.

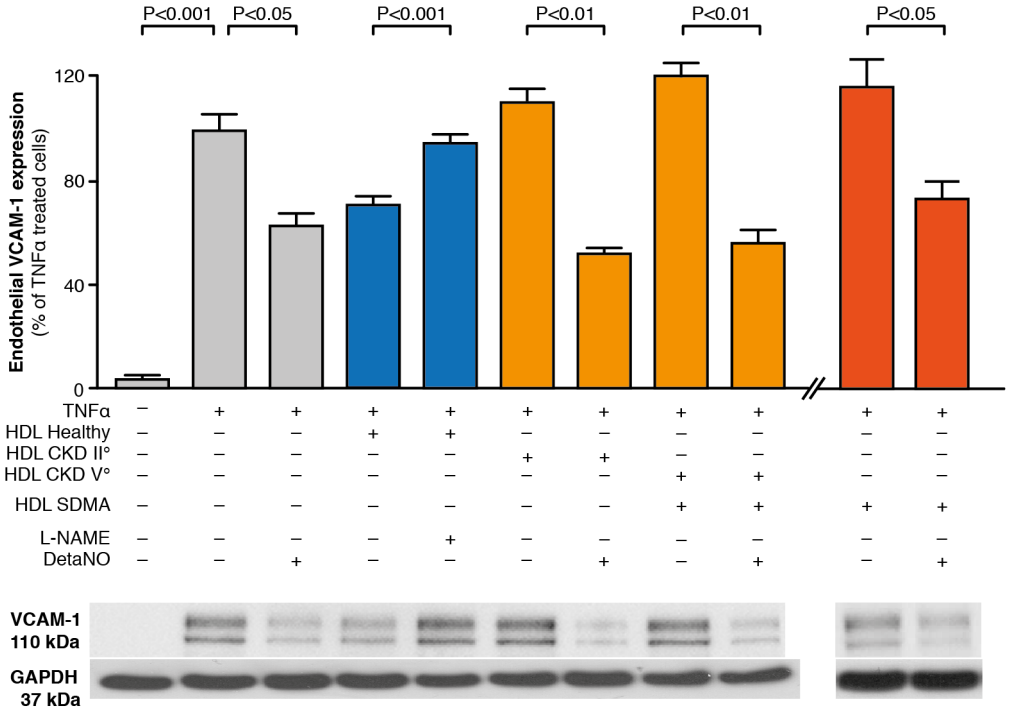

Figure 46 - Effect of *HDL*^{Healthy}, *HDL*^{CKD} or *HDL*^{SDMA} (50µg/ml) with or without co-incubation with L-NAME (0.3 mM) and *HDL*^{CKD} or *HDL*^{SDMA} with or without co-incubation with DetaNO (1 mM) on TNFα-induced (5 ng/ml) endothelial VCAM-1 expression (n=3-6 per group).

Taken together, these results clearly suggest that a reduced endothelial NO bioavailability induced by *HDL*^{CKD} and *HDL*^{SDMA} represents a major mechanism explaining adverse endothelial effects of modified HDL.

6 Discussion

The results of the present project demonstrate for the first time that HDL from children and adult patients with CKD substantially inhibits endothelial NO production and thereby increases ABP. We have identified accumulation of SDMA in HDL from subjects with even mild CKD as the culprit converting HDL into a noxious particle. Furthermore, we have shown that this abnormal HDL activates endothelial TLR-2 via a novel TLR-1- and TLR-6-co-receptor independent alternative pathway impairing endothelial repair and enhancing endothelial pro-inflammatory activation.

6.1 Effects of HDLCKD on endothelial NO production

We describe the effects of HDL from patients with CKD as a population with a particularly high cardiovascular morbidity and mortality (Go, Chertow et al. 2004; Van Biesen, De Bacquer et al. 2007).

In healthy individuals, endothelial NO production stimulated by HDL serves as an important stimulus to preserve endothelial function (Yuhanna, Zhu et al. 2001; Spieker, Sudano et al. 2002; Bisoendial et al. 2003; Landmesser et al. 2004; Nofer, van der Giet et al. 2004). It has been demonstrated that HDL from healthy subjects induces endothelial NO production via interaction with the receptors SR-BI, $S1P_3$ as well ABCG-1 (Yuhanna, Zhu et al. 2001; Nofer, van der Giet et al. 2004; Terasaka, Yu et al. 2008). Moreover, infusion of rHDL has been described to restore endothelial function i.e. forearm blood flow during intrarterial infusion of acetylcholine *in vivo* in hypercholesterinemic men (Spieker, Sudano et al. 2002). In addition, it has been demonstrated that HDL as well as sphingosine-1-phosphate (S1P) reduced myocardial injury in an *in vivo* model of myocardial ischemia/reperfusion in mice (Theilmeier, Schmidt et al. 2006). The authors could show that the beneficial effects of HDL in this model are mediated by eNOS.

However, recent evidence suggests that several pathological conditions such as coronary artery disease, diabetes or antiphospholipid syndrome may led to a loss of HDL's stimulatory effect on endothelial nitric oxide synthase (Charakida, Besler et al. 2009; Sorrentino, Besler et al. 2010; Besler, Heinrich et al. 2011). The authors claimed oxidative modifications of Apo-A1 as the underlying mechanism for the altered effect of HDL from these patients on endothelial NO production.

By contrast, in the present study, we found that HDL from patients with CKD almost completely suppressed endothelial NO production in a dose-dependent manner. Importantly, this adverse effect of HDL^{CKD} was already present with HDL from patients with an early stage of CKD. These results may contribute to the high cardiovascular risk, which is already

present in patients with a slightly reduced renal function (Go, Chertow et al. 2004; Van Biesen, De Bacquer et al. 2007).

To evaluate as to whether CKD and not concomitant disease, which is commonly found in adult patients with CKD, we subsequently analyzed the effect of HDL from children with CKD on endothelial NO production. Children with CKD already exhibit an increased risk for cardiovascular events (Kavey et al. 2006; Shroff et al. 2009; Shroff et al. 2011). However, in contrast to adult patients, CKD in children is mainly caused by hereditary kidney disorders or glomerulonephritis and not by diabetes or hypertension, which represent the main causes for CKD in adults. Notably, HDL from children with CKD also inhibited endothelial NO production indicating that the CKD itself and not concomitant diseases is responsible for the observed adverse effects of HDL.

eNOS, the NO generating enzyme in endothelial cells is crucially involved in the regulation of vascular tone. Studies in knock-out mice have clearly documented that genetic defiency of the eNOS gene leads to an increased blood pressure and promotes the formation of atherosclerotic lesions (Huang et al. 1995; Shesely et al. 1996; Chen et al. 2001; Kuhlencordt et al. 2001). Moreover, it has been shown that endothelial activation of the $S1P_3$ receptor by HDL reduces the arterial blood pressure in rodents (Nofer, van der Giet et al. 2004). To evaluate the effect of HDL from CKD patients on arterial blood pressure, we injected HDL from healthy subjects or patients with CKD into mice and measured ABP. Indeed, we observed that infusion of HDL^{CKD} into mice significantly increased ABP. This observation is of overriding importance, since hypertension is a strong promoter of cardiovascular disease and frequently observed in patients with CKD (Peterson et al. 1995). Thereby, hypertension does not only present a risk factor for cardiovascular disease in CKD patients, but also a risk factor for the progression of CKD itself (Peterson, Adler et al. 1995; Jafar et al. 2003; Kunz et al. 2008). Thus, our results indicate that HDL may be involved into a vicious circle of hypertension and CKD.

Next, we aimed to assess the underlying mechanism responsible for the reduction of endothelial NO bioavailability by HDLCKD. Therefore, we quantified endothelial production of ROS in response to HDL. ROS are known to be directly involved in the pathogenesis of atherosclerotic diseases (Cai et al. 2000; Griendling et al. 2000; Harrison et al. 2003; Forstermann et al. 2006). Moreover, ROS can react with NO forming peroxynitrite and thereby reduce endothelial NO bioavailability (Pacher, Beckman et al. 2007). Indeed, HDL from patients with CKD potently induced endothelial superoxide production, while HDL from healthy subjects had no affect on endothelial superoxide production.

In contrast to previous reports describing “dysfunctional HDL”, we could demonstrate that HDL^{CKD} not only loses its vaso-protective properties but rather changes into a noxious particle strongly promoting endothelial dysfunction and hypertension.

6.2 SDMA accumulates in the HDL particle and alters its endothelial effects

We hypothesized that methylarginines, which accumulate when renal function deteriorates, may associate with the HDL particle from CKD patients and affect its vasoprotective properties.

ADMA, a methylation product of L-arginine residues, is an endogenous eNOS inhibitor increased in patients with CKD (Boger et al. 1998; Zoccali et al. 2001; Kielstein et al. 2002; Fliser et al. 2005). Moreover, it has been demonstrated that infusion of ADMA into healthy subjects increases arterial blood pressure, reduces renal plasma flow and cerebral blood flow (Kielstein et al. 2004; Kielstein et al. 2006). However, by using a newly modified mass spectrometry approach, we ruled out the presence of ADMA in the HDL fraction.

Surprisingly, we detected substantial amounts of SDMA, an isomer of ADMA, in HDL^{CKD} but not in $HDL^{Healthy}$. We and others have previously demonstrated that SDMA serum concentrations are elevated in several clinical conditions such as CKD and pulmonary arterial hypertension (Kielstein, Boger et al. 2002; Pullamsetti et al. 2005; Schepers et al. 2011). Although SDMA is thought to be functionally inactive, several studies highlighted its predictive value for cardiovascular events (Bode-Boger et al. 2006; Meinitzer et al. 2011). However, recent trials have indicated a functional role of SDMA in CKD-associated cardiovascular disease. Incubation of endothelial cells with SDMA for 24 hours slightly reduced endothelial NO production and increased endothelial ROS production (Bode-Boger, Scalera et al. 2006). Moreover, it has been shown that SDMA may induce production of ROS and proinflammatory cytokines in mononuclear cells (Schepers et al. 2009; Schepers, Barreto et al. 2011).

In order to examine as to whether incorporation of SDMA into the HDL particle may alter the ability of HDL to enhance the endothelial NO bioavailability, we supplemented HDL from healthy subjects with SDMA and measured NO production. Importantly, supplementation of HDL with SDMA in concentrations as measured in patients with CKD resulted in deleterious effects of HDL^{SDMA} on endothelial NO production.

The enzymatic activity of eNOS is tightly regulated by Akt-dependent phosphorylation of distinct amino acid residues in the eNOS molecule. While phosphorylation at Serine 1177 stimulates eNOS enzymatic activity, phosphorylation at Threonine 495 leads to an inhibition of eNOS activity. HDL has been shown to promote eNOS-dependent NO production by

stimulating phosphorylation of eNOS (Yuhanna, Zhu et al. 2001; Mineo, Yuhanna et al. 2003; Drew et al. 2004; Nofer, van der Giet et al. 2004; Assanasen, Mineo et al. 2005; Terasaka, Yu et al. 2008).

We therefore studied the effect of HDLCKD as well HDLSDMA on Akt-dependent eNOS phosphorylation in endothelial cells. In line with previous reports, we could document that HDLHealthy stimulated Akt-dependent phosphorylation of eNOS at Serine 1177 residue. In contrast, HDLCKD as well as HDLSDMA, both, reduced phosphorylation of Akt at Serine 473 and enhanced phosphorylation of eNOS at Threonine 495 residue. These results indicate that HDL from patients with CKD and HDL supplemented with SDMA reduce eNOS enzymatic activity by modulating the regulatory phosphorylation of eNOS.

6.3 SDMA associates with apolipoprotein A-I in the HDL particle

Since supplementation of LDL with SDMA did not change its effect on endothelial NO production, we hypothesized that SDMA may be associated to the protein part of the HDL complex. Indeed, we observed that rHDL and Apo-A1 supplemented with SDMA reduced endothelial NO production, which indicates that SDMA may associate to Apo-A1. These findings indicate that the presence of SDMA in the HDL particle may represent a novel mechanism leading to deterioration of HDL's vascular effects and its transformation into a pro-atherogenic particle.

6.4 Abnormal HDL interacts with TLR-2

$HDL^{Healthy}$ exerts its endothelial-atheroprotective effects by binding to the endothelial scavanger receptor SR-BI or S1P3 receptors as well as by activating ABCG-1 (Acton, Rigotti et al. 1996; Yuhanna, Zhu et al. 2001; Nofer, van der Giet et al. 2004; Assanasen, Mineo et al. 2005; Terasaka, Yu et al. 2008). Our data indicate that supplementation with SDMA does not change the affinity of HDL to the receptor SR-BI. Therefore, we focused on receptors, which are associated with abnormal endothelial activation. TLR-2 and TLR-4 are both expressed on endothelial cells (Edfeldt et al. 2002; Grote et al. 2010) and known to be activated by microbial lipoprotein patterns (Takeuchi and Akira 2010). Indeed, activation of TLR1/TLR-2, TLR-2/TLR-6 or TLR-4 by microbial lipoproteins initiates defense mechanisms of the innate immunity against infectious pathogens (Brightbill et al. 1999; Roux et al. 2011). Interestingly, it has been shown that TLR-2 may also play an important role in development of atherosclerosis (Mullick et al. 2005; Favre et al. 2007; Mullick et al. 2008). Notably, in the present study, the selective inhibition or genetic deficiency of TLR-2, but not TLR-4, almost

completely abrogated the adverse effects of modified HDL on endothelial NO bioavailability, endothelial repair and ABP. Recent evidence suggests, that circulating cells of the lymphoid and myeloid compartment are crucially involved in the pathogenesis of hypertension (Harrison et al. 2011). Blood pressure measurements using WT and *Tlr2*$^{-/-}$ bone marrow chimeras suggest that TLR-2 expression on endothelial cells, rather than circulating mononuclear cells, mediates the adverse effect of *HDL*^{CKD} on ABP. This suggests that the presence of SDMA transforms HDL towards a phenotype that activates this innate immune receptor on the endothelium.

6.5 Activation of endothelial TLR-2 induces endothelial dysfunction

To further understand the functional implications of endothelial TLR-2 activation, we studied the effects of TLR2 on distinct endothelial cell functions. We showed for the first time, that activation of TLR-2 by its agonists Pam3CSK4 and FSL-1 reduces endothelial NO release and that injection of Pam3CSK4 into mice induces an increase of ABP in a TLR-2-dependent manner.

6.6 Abnormal HDL induces a novel NF-κB independent pathway of TLR-2 in endothelial cells

It is well known that activation of TLR-1/TLR-2 and TLR-2/TLR-6 by their classical bacterial ligands induces the NF-κB-dependent production of cytokines and other proinflammatory molecules in a variety of cell types (Alexopoulou, Thomas et al. 2002; Fujita, Into et al. 2003; Sandor, Latz et al. 2003; Beutler 2004).

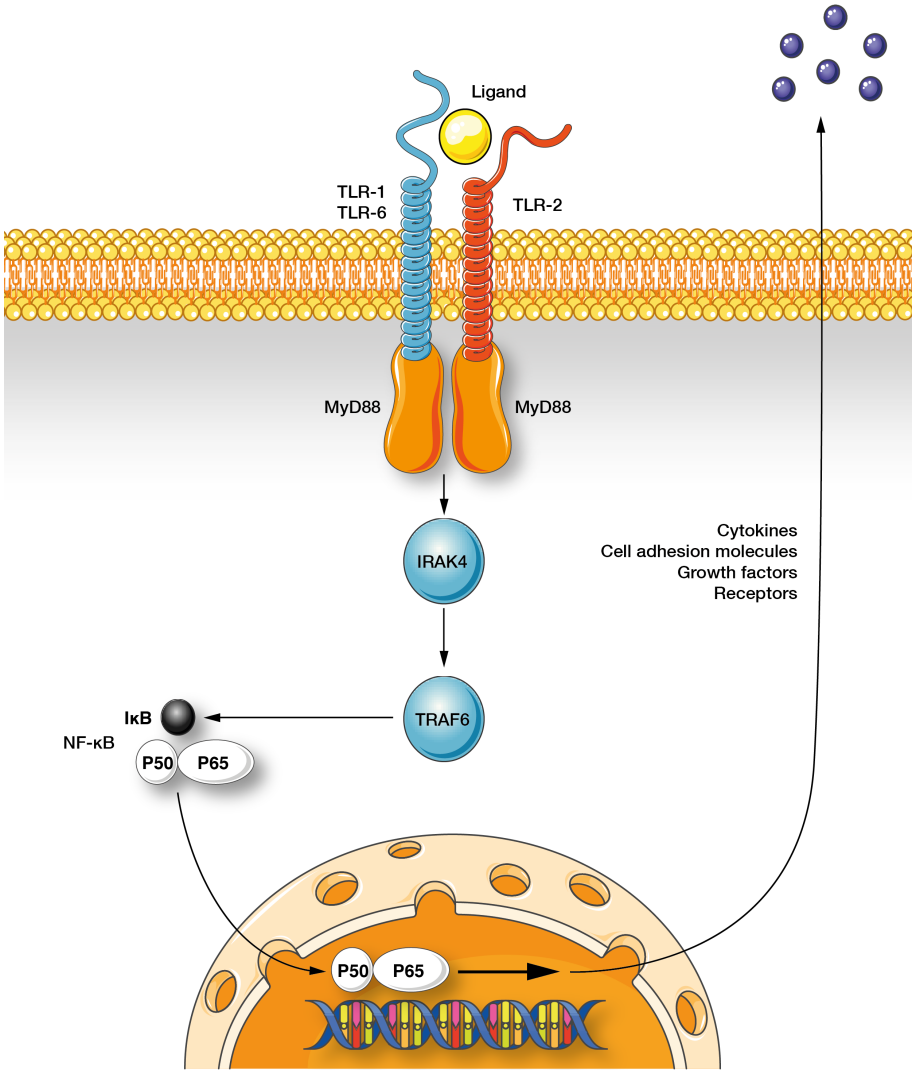

Figure 47 – Classical signaling of TLR-2 in mononuclear cells.

Surprisingly, we observed that abnormal HDL did not induce TLR-1/TLR-2 or TLR-2/TLR-6-mediated NF-κB activation and subsequent cytokine release in endothelial cells or PBMC. Notably, blockade of TLR-1 and/or TLR-6 did not affect endothelial NO production and superoxide production in response to classical TLR-2 ligands or HDL^{CKD} and HDL^{SDMA}. Thus, we were able to document that the effect of TLR-2 activation on endothelial NO production is NF-κB independent. In fact, HAEC lack TLR-1 and TLR-6 on the cell surface and therefore do not classically respond to bacterial antigens.

To determine the molecular mechanisms on how endothelial TLR-2 activation may inhibit endothelial nitric oxide production, we could document that TLR-2 activation by its ligand Pam3CSK4 - similar to HDL^{CKD} and HDL^{SDMA} – did not stimulate Akt-dependent eNOS-stimulating phosphorylation, but and enhanced eNOS-inhibiting phosphorylation. It has recently been shown that TLR-2 activation can induce ROS production in monocytes and

macrophages (West et al. 2010; Beaulieu, Lin et al. 2011; West et al. 2011). In the present study, we could demonstrate that endothelial activation of TLR-2 induces endothelial ROS production. Of note, TLR-2-induced ROS production was abolished after TLR-2 and not TLR-1 or TLR-6 blockade. This indicates that TLR-2 stimulates endothelial ROS production thereby inhibiting NO bioavailability via a novel TLR-1- and TLR-6-co-receptor independent alternative pathway. Furthermore, we demonstrate that the TLR-2 induced NF-κB-dependent cytokine release and the TLR-2 induced ROS production are two distinct pathways, which can be independently targeted by TLR-2 activating agents. In addition, we showed that TLR-2 induced endothelial ROS production is inhibited by blocking NADPH oxidase, identifying NADPH oxidase as prevailing source of ROS after TLR-2 activation via SAPK/JNK, which has been previously shown to stimulate vascular superoxide production *in vitro* and *in vivo* (Osto, Matter et al. 2008; Shi, Cosentino et al. 2011).

6.7 Effects of HDLCKD as well as HDLSDMA on endothelial repair and inflammation

Additionally, we assessed the effect of HDL from CKD patients on endothelial repair and inflammation. We could newly document that HDL^{CKD} loses its ability to promote endothelial migration and, *in vivo*, the repair of endothelial lesions in the perivascular carotid injury model. Moreover, HDL^{CKD} as well as HDL^{SDMA}, both, did neither suppress TNF-α stimulated endothelial VCAM-1 expression nor adhesion of mononuclear cells to a TNF-α activated endothelial monolayer. In contrast, $HDL^{Healthy}$ reduced endothelial proinflammatory activation by reducing endothelial VCAM-1 expression and, subsequently, endothelial mononuclear cell adhesion. Notably, both processes, endothelial repair as well as endothelial proinflammatory activation have been previously shown to dependent on endothelial NO bioavailability (Kubes, Suzuki et al. 1991; Pacher, Beckman et al. 2007; Sorrentino, Bahlmann et al. 2007). These results underscore the impact of an inhibition of the endothelial NO production on distinct endothelial properties of HDL.

6.8 Implications for HDL-targeting therapies

Currently, HDL-targeting therapies are in the focus of clinical cardiovascular research. Notably, raising HDL-cholesterol (HDL-C) serum levels in non-CKD patients by using Niacin in addition to Statin treatment has not significantly reduced the percentage of patients reaching the primary cardiovascular endpoint[6].

Inhibition of CETP has been recognized as a potent approach to elevate HDL-C serum levels. Treating patients with the CETP inhibitor Torcetrapib increased HDL-C serum levels by 72.1 % after 12 months of treatment[4]. Surprisingly, Torcetrapib treatment significantly augmented the systolic blood pressure leading to higher rate of cardiovascular events in these patients. This effect of Torcetrapib has been mainly attributed to an off-target effect of Torcetrapib to increase aldosterone secretion. Therefore, other CETP inhibitors without off-target effects such as Dalcetrapib have been evaluated. Although Dalcetrapib reliably increased HDL-C levels, the investigators of the DAL-HEART program could not document any significant reduction of the incidence of the primary endpoint as compared to placebo treated subjects[37]. Moreover, Dalcetrapib treatment did not improve the flow-mediated vasodilation as a surrogate for the endothelial NO bioavailability[26]. These results clearly indicate that simple raising of HDL-C serum levels does not automatically entail an amelioration or restoration of HDL's atheroprotective properties. Although these aforementioned studies have not been performed in patients with CKD, it remains more than questionable as to whether an HDL-increasing therapy would be beneficial in CKD patients. In particular, if fundamental changes of the composition and function of HDL are present, as intriguingly documented for HDL of CKD patients.

7 Conclusion

Taken together the present study provides several important novel insights. First, we showed that even mild CKD inverts HDL's endothelial-protective properties. In fact, HDL^{CKD} induces endothelial dysfunction, impairs endothelial repair and increases ABP. Second, we identified SDMA in HDL^{CKD} as the culprit that modifies HDL to induce these deleterious endothelial effects. Moreover, we demonstrate that such modified HDL activates endothelial TLR-2 signaling resulting in enhanced ROS production and inhibition of endothelial NO bioavailability. Third, our data provide evidence that these effects are mediated by activation of TLR-2 via a novel TLR-1- or TLR-6-co-receptor-independent alternative pathway. These data illustrate as to how a small compound like SDMA can modify the HDL particle mimicking a damage-associated molecular pattern to activate TLR-2, thereby linking innate immunity, endothelial dysfunction and arterial hypertension. These observations may also have important clinical implications for HDL-raising therapies in patients with CKD, which have not yet proven to be beneficial so far.

The findings of the present study are summarized in the following figure:

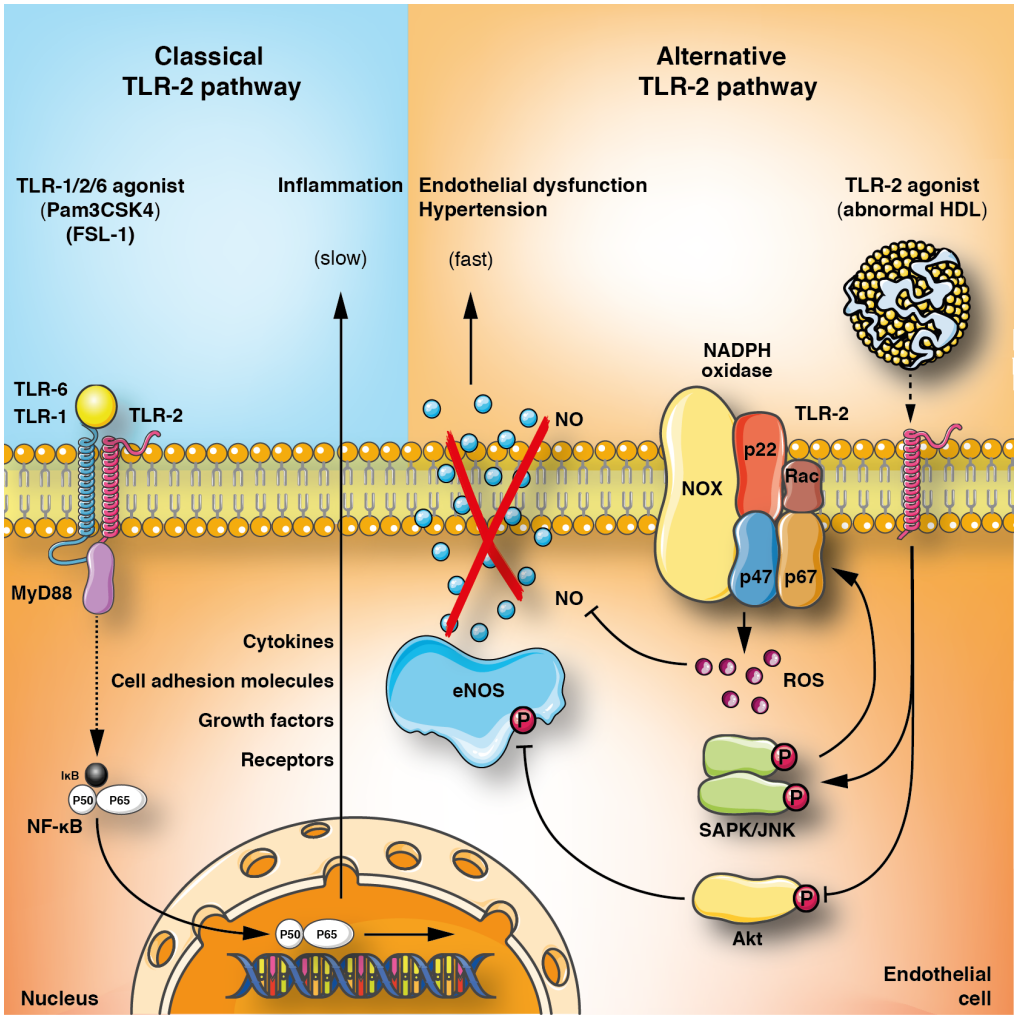

Figure 48 – Graphical summary of the results

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9 Acknowledgments

At first I would like to thank my supervisor Prof. Dr. Ulf Landmesser for his support. Moreover, I thank Prof. Dr. Thomas F. Lüscher for the opportunity to work in his laboratory and his very constructive comments.

Many thanks to Dr. Lucia Rohrer and Prof. Dr. Arnold von Eckardstein for their excellent technical and personal support. In particular to Lucia, who always lend me her ear in times of trouble.

I thank Prof. Dr. Olivier Devuyst for being the responsible faculty member of my thesis committee and for his constructive support.

In particular, I would like thank Prof. Dr. Danilo Fliser for giving me the opportunity to conduct this ambitious project. During many difficulties, he always had confidence in me and the project.

A big thanks goes to all my friends in Zurich for their support and the nice time we spent together: Nicolle, Kira, Maja, Tine, Stephan, Remo, Dominique, Francesco, Yi, Giovanni, Alexander, Eric, Helen. My special thanks goes to Stephan Keller, who always gave me a lot of support and helped me in so many things. Many thanks for ESC replacement days and times in the wine cellar or the invitations. Despite all “deep hits”, I will not forget these nice years in Zurich.

Moreover, I would like to thank my friends and my research group in Germany: Stephen, Inga, Anne, Sarah, Claudia, Gabriel, Andreas and my doctoral students.

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